REMARKS

Claims 1-3 and 5-14 are pending. Claim 4 is cancelled without prejudice to the prosecution of its subject matter in other patent applications. Neither the amendments nor the new claims constitute new matter.

Claims 1-2 and 4-11 are rejected under the first paragraph of 35 U.S.C. § 112, because, according to the Examiner, the specification does not enable any person skilled in the art to which the invention pertains, or with which it is most nearly connected, to make and use the invention commensurate with the scope of these claims.

Claims 1-11 are rejected under 35 U.S.C. § 103(a) as being rendered obvious by Taylor *et al.* (Taylor *et al.*, Blood 1997;89:4078-4084) and Coller *et al.* (Coller *et al.*, Haemostasis 1996;26:285-293) in view of Friedlander *et al.* (Proc. Natl. Acad. Sci. USA 1996;93:9764-9769) and Brooks *et al.* (United States Patent No. 5,753,230).

Applicants respectfully traverse the Examiner's rejections of the aforementioned claims for the reasons set forth below.

I. The Claims Are Enabled Under The First Paragraph Of 35 U.S.C. § 112

Claims 1-2 and 4-11 are rejected under the first paragraph of 35 U.S.C. § 112 because, according to the Examiner, the specification does not enable any person skilled in the art to which the invention pertains, or with which it is most nearly connected, to make and use the invention commensurate with the scope of these claims. Specifically, the Examiner contends that the specification is enabling for methods of administering monoclonal antibodies which bind to the integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ wherein the monoclonal antibody is 7E3 or a mouse/human chimera thereof, but is not enabling for methods employing any other antibodies. The Examiner contends that the 7E3 antibody has the unique property of binding and acting as an antagonist of both $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins, citing, as contrasting examples, 10E5 (which binds to both integrins but is not an antagonist) and LM609 (which binds to and antagonizes $\alpha_v\beta_3$ but not $\alpha_{IIb}\beta_3$).

In response, Applicants repeat arguments offered in the parent of the instant application, that methods of identifying molecules that bind to and antagonize the recited integrins were standard techniques known in the art, such that no invention would

need to be made in order to identify an immunoglobulin or fragment thereof that antagonizes $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$.

There is no reason to conclude that 7E3 is the only possible antibody to combine binding and antagonism to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins. The fact that AP3 and LM609, both cited in the instant application, lack both properties is not relevant to the likelihood of finding both activities in a single antibody; AP3 and LM609 were used as controls in the working examples and would be expected to differ in their functionalities.

In addition, to advance the prosecution of the claims, claim 1 has been amended to incorporate a further set of characteristics, as recited in original claim 4, providing further criteria to identify immunoglobulins and immunoglobulin fragments which may be used according to the invention. This amendment is made without prejudice to the prosecution, in other patent applications, of subject matter excluded from the claim by amendment.

For the foregoing reasons, it is requested that the rejection of the claims as unenabled be removed.

II. The Claims Are Not Obvious Under 35 U.S.C. § 103(a)

The Examiner has rejected Claims 1-11 under 35 U.S.C. § 103(a) as being rendered obvious by Taylor *et al.* (Blood 1997;89:4078-4084; "Taylor") and Coller *et al.* (Haemostasis 1996;26:285-293; "Coller") in view of Friedlander *et al.* (Proc. Natl. Acad. Sci. USA 1996;93:9764-9769; "Friedlander")) and Brooks *et al.* (United States Patent No. 5,753,230; "Brooks"). Specifically, the Examiner asserts that Coller teaches that 7E3 and related molecules inhibit $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$; that Taylor teaches the use of 7E3 and related molecules, in the claimed dosages and routes of administration, for protection of baboons against microangiopathic hemolytic anemia and microvascular thrombotic renal failure; that Friedlander teaches the involvement of $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ in angiogenesis and macular degeneration and the use of integrin antagonists as anti-angiogenic agents; and that Brooks teaches that angiogenesis is important in a wide range of clinical diseases, and that inhibitors of $\alpha_v\beta_3$ can inhibit angiogenesis. Thus, according to the Examiner, one of ordinary skill would be able to combine the teachings of Taylor *et al.* with the other

teachings cited above to extend the findings of Taylor *et al.* to cover the treatment of angiogenesis and related diseases.

Applicants assert that the claims are not obvious over the cited references, because there is no basis to combine the cited references to achieve the claimed invention. Moreover even if, for the sake of argument, the references were combined, they would teach *against* the claimed invention.

The disclosure of Taylor would not provide any motivation to the skilled artisan to use the 7E3 antibody, or any other antibody having antagonism to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, to inhibit angiogenesis in a subject. The diseases addressed in Taylor, diffuse intravascular coagulation, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome are all disorders of the blood coagulation mechanism itself. Taylor expressly states the reason for using 7E3 antibody:

> Since platelets have been implicated in mediating at least in part the tissue damage associated with all three of these syndromes, we evaluated the effect of treating animals with a potent antiplatelet agent, the F(ab')2 fragment of the murine monoclonal antibody (MoAb) 7E3, which binds to platelet glycoprotein (GP) IIb/IIIa receptors and inhibits platelet aggregation.

Taylor concerns the treatment of coagulation disorders with an antibody known to inhibit platelet-mediated coagulation. There would be no motivation to look, from Taylor, to angiogenesis-related references.

For example, there would be no reason to look to Friedlander, which deals with inhibition of angiogenesis in retinal disorders. Friedlander uses a cyclic peptide antagonist of $\alpha_v\beta_3$, and does not teach the use of monoclonal antibodies. Moreover, it does not relate to antagonism of $\alpha_{IIb}\beta_3$ on platelets. There would be no reason to combine Taylor with Friedlander.

Brooks, while teaching the use of monoclonal antibodies, relates to inhibition of angiogenesis by antagonism of $\alpha_v\beta_3$, and teaches *against* the use of agents that also inhibit $\alpha_{IIb}\beta_3$ (at column 3, lines 22-25):

> In a particularly preferred embodiment, the $\alpha_v\beta_3$ antagonist . . . does not substantially inhibit binding of fibrinogen to $\alpha_{IIb}\beta_3$.

As Taylor requires antagonism of $\alpha_{IIb}\beta_3$, there would be no motivation to combine Taylor with Brooks, as Taylor uses 7E3 for its $\alpha_{IIb}\beta_3$ antagonism properties.

Coller relates to the anti-thrombotic effects of $\alpha_{IIb}\beta_3$ antagonists, including 7E3.

Applicants respectfully contend that the Examiner has used Taylor to supply antagonism of $\alpha_{IIb}\beta_3$ to the Friedlander and Brooks references, using Coller as a general reference for 7E3, when there is no other logical reason to combine them. A combination of prior art features will only be deemed obvious if the prior art references contain a suggestion for so combining their teachings; the hindsight afforded by the invention cannot be used to negate its insight. *In re Fine*, 837 F.2d 1071, 1075 (Fed.Cir.1988).

Further, if the references were, for the sake of argument, combined, the sum disclosure would teach away from the claimed invention, because Brooks teaches that $\alpha_v\beta_3$ antagonists preferably lack anti-$\alpha_{IIb}\beta_3$ activity.

Applicants further invite the Examiner's attention to the following post-effective filing date (June 4, 1998) publications, which are also listed in the accompanying Supplemental Information Disclosure Statement and PTO 1449 form.

First, Coller, "Potential non-glycoprotein IIb/IIIa effects of abciximab," Am. Heart J. 1999; 138:S1-S5 ("Coller 2"; Exhibit A), discloses that in addition to binding to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, 7E3 also binds to an activated form of the $\alpha_M\beta_2$ receptor. This publication further includes the statement:

> Animal model data support the potential novel utility of $\alpha_v\beta_3$ blockade in other disease states, such as sickle cell disease, and the inhibition of tumor angiogenesis, raising the possibility that abciximab may be useful for preventing sickle cell adhesion and inhibiting tumor angiogenesis. However, even if these preliminary animal data are confirmed in humans, it will remain to be established whether abciximab, with its additional glycoprotein IIb/IIIa [$\alpha_{IIb}\beta_3$] blockade effect, will prove more beneficial than selective $\alpha_v\beta_3$ blockade.

A slightly later publication, Cohen et al., "Potential Future Clinical Applications for the GPIIb/IIIa Antagonist, Abciximab in Thrombosis, Vascular and

Oncological Indications," Pathol. Oncol. Res. 2000; 6(3):163-174 ("Cohen"; Exhibit B) discusses the rationale for an anti-angiogenic effect of antagonism to GPIIb/IIIa ($\alpha_{IIb}\beta_3$)[1]:

> In addition to facilitating hematogenous metastasis, platelets may also participate in angiogenesis and growth of primary and disseminated tumors. Pinedo and Folkman have postulated that a true anti-angiogenic therapy must target platelets.[2] Platelets contain one of the largest stores of angiogenic and mitogenic factors, and with a circulating half-life of ~5-7 days [citation], they could provide tumors with a continuous supply of growth factors. Tumor vasculature is leaky and extravasated fibrin(ogen) that is deposited on the tumor surface can provide an ideal substrate for platelet binding. Platelet granules contain a variety of factors such as VEGF, PDGF, TGF-β, and fibrinogen, and these modulators are immediately secreted after platelet activation. Abciximab can block platelet aggregation and adhesion to fibrin(ogen), and it also inhibits platelet degranulation. By blocking granule release, abciximab inhibits secretion of serotonin, TGF-β, PDGF AB[3] and VEGF.[4] Most of these factors have been implicated in various steps of tumor progression and metastasis. VEGF is one such angiogenic factor that is stored in large amounts in circulating platelets. Abciximab inhibits ADP-stimulated platelet secretion of VEGF.[5] In addition, tumor cells also induce platelets to secrete VEGF and this secretion is also blocked by abciximab.[6] Blockade of VEGF secretion by abciximab is due to its ability to inhibit both platelet aggregation and tumor-cell platelet binding that is mediated by $\alpha_v\beta_3$ and platelet GPIIb/IIIA [$\alpha_{IIb}\beta_3$]. It is tempting to speculate that when administered to patients with cancer, abciximab could directly block $\alpha_v\beta_3$ and GPIIb/IIIa [$\alpha_{IIb}\beta_3$] function and indirectly block VEGF function. This multi-receptor binding of abciximab may distinguish it from other anti-angiogenic antagonists that are unable to inhibit platelet GPIIb/IIIa. However, the safety of this dual effect of abciximab remains to be defined.

Trikha et al., ""Platelets and Cancer: Implications for Antiangiogenic Therapy," Seminars in Thrombosis and Hemostasis 2002; 28:39-44 ("Trikha 1"; Exhibit C), while continuing to express caution regarding use in human patients, further discloses the observation that platelets, in *in vitro* models of angiogenesis, stimulate sprouting of endothelial cells that can be blocked by GPIIb/IIIa ($\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$)

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1 Cohen, paragraph bridging pages 165-166.
2 Cohen citing Pinedo et al., Lancet 1998; 352:1775-1777.
3 Cohen citing Chronos et al., Eur. Heart J. 1999; 1S:E11-E17.
4 Cohen citing Amirkhosravi et al., Platelets 1999; 10:285-292, Möhle et al., Proc. Natl. Acad. Sci. U.S.A. 1997; 94:663-668 and Maloney et al., Am. J. Physiol. 1998; 275:H1054-H1061.
5 Cohen citing Maloney et al., Am. J. Physiol. 1998; 275:H1054-H1061.
6 Cohen citing Amirkhosravi et al., Platelets 1999; 10:285-292.

antagonists.[7] Trikha 1 further refers to experimental evidence that 7E3 has antiangiogenic and antimetastatic effects *in vivo*:

> A few studies suggest that combined blockade of platelet GpIIb/IIIa[$\alpha_{IIb}\beta_3$] and the angiogenic $\alpha_v\beta_3$ integrin is superior to blockade of just $\alpha_v\beta_3$ integrin.[8] Abciximab is distinct from eptifibatide and tirofiban as it binds and blocks both GpIIb/IIIa and $\alpha_v\beta_3$ integrin with equivalent affinity. Recent *in vivo* findings suggest that mE3(F(ab')$_2$ has both antiangiogenic and antimetastatic properties.[9] These studies support the idea that antiplatelet therapy is also antiangiogenic.

Finally, Trikha et al., "Multiple Roles for Platelet GPIIb/IIIa and $\alpha_v\beta_3$ Integrins in Tumor Growth, Angiogenesis, and Metastasis," Cancer Res. 2002; 62:2824-2833 ("Trikha 2"; Exhibit D) reports experiments that show that the antitumor effect of m7E3F(ab')$_2$ was greater when *both* GpIIb/IIIa $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, rather than $\alpha_v\beta_3$ alone, were blocked. Trikha 2 addresses data in which, as in the working examples of the instant specification, LM609 and 7E3 had similar anti-tumor effects in a SCID mouse model. Trikha 2 states:[10]

> Regular administration of LM609 significantly inhibited growth of $\alpha_v\beta_3$ – negative tumors by blocking the growth of human blood vessels. Because LM609 does not cross-react with mouse integrins, its anti-angiogenic activity was attributed to blockade of human $\alpha_v\beta_3$ receptors in the vasculature of the human skin. A subsequent study using the murine IgG equivalent of c7E3 Fab (m7E3 IgG) in the same model achieved similar results as LM609.[11] Similar to LM609, 7E3 does not cross-react with murine integrins; therefore, it inhibited growth of human tumors by blocking human $\alpha_v\beta_3$ in the vasculature of the human skin. In these studies, a partial inhibition of tumor growth was observed, and the combined effect of blocking tumor cell-expressed $\alpha_v\beta_3$ and endothelial cell-expressed $\alpha_v\beta_3$ was not evaluated.. . . One purpose of our study was to evaluate whether combined blockade of host and tumor cell-expressed integrins was superior to blockade of tumor cell-expressed integrins *in vivo*.

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7 Trikha 1 at page 41, citing Trikha et al., Proc. Am. Assoc. Cancer Res. 2000; 42:824 Abstr. 3678.

8 Trikha 1 at page 42, citing Trikha et al., Proc. Am. Assoc. Cancer Res. 2000; 42:824 Abstr/ 3678.

9 *Id.*

10 Trikha 2 at page 2824.

11 Trikha 2, citing Varner et al., Angiogenesis 1999; 3:53-60.

Specifically, Trikha 2 reports that in their experiments, in SCID mice, m7E3F(ab')$_2$ only partially inhibited the growth of human melanoma tumors. "Because c7E3 and m7E3F(ab')$_2$ do not cross-react with murine integrins, this inhibition of metastasis and tumor growth is attributable to direct blockade of human $\alpha_v\beta_3$ integrins."[12] In contrast, in nude rats, "where m7E3F(ab')$_2$ simultaneously binds to both human tumor and host platelet GPIIb/IIIa[$\alpha_{IIb}\beta_3$] and endothelial $\alpha_v\beta_3$ integrins" m7E3F(ab')$_2$ "completely blocked human tumor formation and growth of human melanoma tumors."[13] Trikha 2 concludes that:

> In this rat xenograft model, which mimics the clinical situation, combined antiangiogenic and antitumor activity of m7E3F(ab')$_2$ was superior at inhibiting tumor growth when compared with its antitumor activity in the mouse xenograft model.

Thus, post-effective filing date publications indicate that in contrast to the combined teaching of the cited references, which teach that antagonism to $\alpha_{IIb}\beta_3$ is detrimental, the dual antagonism toward $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, in particular as exhibited by 7E3, has improved anti-angiogenic activity.

Therefore, for all the foregoing reasons, the references do not render the claims obvious, and the rejection should be withdrawn.

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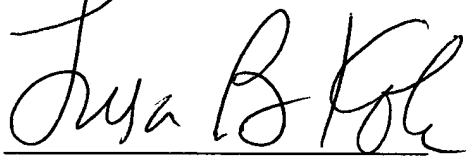
12 Trikha 2, Abstract.
13 *Id.*

CONCLUSION

Based on the foregoing remarks and in light of the amendments, Applicants submit that the present application is in condition for allowance. A Notice of Allowance is therefore respectfully requested.

If a telephone interview would be of assistance in advancing the prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

Respectfully submitted,
BAKER BOTTS L.L.P.



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Potential non–glycoprotein IIb/IIIa effects of abciximab

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The antithrombotic effect of abciximab is believed to be primarily due to its blockade of platelet glycoprotein IIb/IIIa receptors, leading to the inhibition of platelet aggregation. Studies have, however, identified that antibody 7E3, the parent molecule of abciximab, and/or abciximab itself, binds to both "activated" αMβ2 receptors and αVβ3 receptors. Because αMβ2 receptors are present on granulocytes and monocytes, cells that have been implicated in contributing to atherosclerosis, intimal hyperplasia after vascular injury, reperfusion injury, and thrombin generation, it is possible that some of abciximab's effects relate to this reactivity. Similarly, because αVβ3 has been implicated in platelet adhesion to osteopontin, intimal hyperplasia after vascular injury, and platelet-mediated thrombin generation, it is possible that some of abciximab's beneficial effects relate to this reactivity. Blockade of αVβ3 receptors may also be beneficial in other disease states because, in animal models, such blockade inhibits tumor angiogenesis and sickle cell adhesion to blood vessel endothelium. Despite these intriguing observations, there are no direct data to support any beneficial roles or any unwanted side effects related to the reactivities of abciximab with "activated" αMβ2 or αVβ3 receptors. (Am Heart J 1999;138:S1-S5)

The murine monoclonal antibody 7E3 and all of its derivatives that have been used for in vivo studies (7E3-F[ab´]$_2$, 7E3 Fab´, chimeric 7E3 Fab [c7E3 Fab; abciximab (ReoPro)]) bind to the platelet glycoprotein IIb/IIIa receptor and inhibit platelet aggregation induced by all of the physiologic and pathologic agonists thought to act in vivo.[1,2] This glycoprotein IIb/IIIa antagonist activity was the basis of its development as an antiplatelet agent to prevent and treat platelet-mediated thrombosis in the cardiovascular system. In the course of its development, however, it was discovered that 7E3 also reacts with 2 other integrin receptors: the αVβ3 "vitronectin" (CD51/CD61) receptor[3,4] and an activated form of the αMβ2 (Mac-1; CD11b/CD18) receptor[5] (Figure 1). The glycoprotein IIb/IIIa and αVβ3 receptors share a common β subunit (glycoprotein IIIa or β3) and thus are from the same integrin subfamily, whereas αMβ2 is from another subfamily. The basis of the crossreactivity of 7E3 with these 3 different receptors is not known with certainty, but a common motif termed a metal ion–dependent adhesion site structure has been identified in both the β3 subunit[6-8] and the inserted domain of the αM subunit.[9] Recently Plescia et al[9] identified peptides in the αM metal ion–dependent adhesion site domain to which 7E3 can bind.

Because the αVβ3 and αMβ2 receptors have been implicated in a number of different physiologic and pathologic processes, it is appropriate to consider whether any of the observed effects of abciximab are due to these additional reactivities and whether the effects of abciximab on these other receptors either pose concerns about toxicity or provide new therapeutic opportunities.

The αMβ2 receptor

The αMβ2 receptor is present on granulocytes, monocytes, and natural killer cells.[10] It can undergo a conformational change when these cells are stimulated with several different agonists, including adenosine diphosphate and the bacterial peptide formyl-methione-

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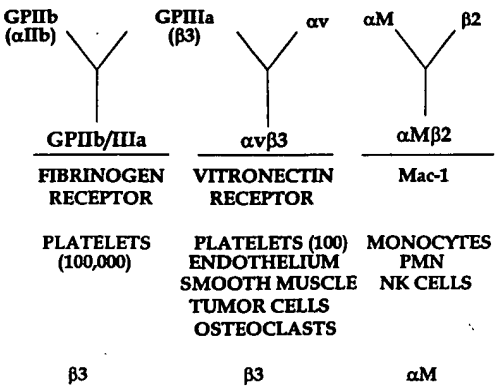
Supported in part by grants 19278 and 54469 from the National Heart, Lung and Blood Institute. Dr. Coller is an inventor of abciximab and, in accordance with federal law and the Patent Policy of the Research Foundation of the State University of New York, shares in royalties paid to the Foundation for the sale of abciximab.
Reprint requests: Barry S. Coller, MD, Department of Medicine, Box 1118, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

0002-8703/99/$8.00 + 0 4/0/99078

Figure 1



Reported reactivities of antibody 7E3. The reaction of αMβ2 requires activation of the αMβ2-containing cells.

leucine-phenylalanine.[5] Several ligands bind to αMβ2, including the complement opsonin, iC3b; the cell-associated counter-receptor, ICAM-1; fibrinogen; coagulation factor Xa; and a ligand on neutrophils.[11-13] αMβ2 contributes to a number of important biologic phenomena including adhesion to and transmigration across endothelium and epithelium, neutrophil aggregation, and neutrophil chemotaxis and binding to and phagocytosis of opsonized particles.[10,13]

Monocytes have been implicated in contributing to atherosclerosis and intimal hyperplasia after vascular injury, and the αMβ2 receptor may be important in mediating the contributions of monocytes to these phenomena.[14-16] Moreover, αMβ2 expression on monocytes and neutrophils is increased at the time of myocardial infarction and remains elevated for at least 1 week.[17] A number of in vitro studies conducted by Altieri and Edgington,[5] Plescia et al,[9] and Simon et al[18] demonstrated that 7E3 can inhibit the interactions of activated monocyte-like cells with known αMβ2 ligands and the adhesion of such cells to injured rabbit blood vessels. Granulocytes have been implicated in reperfusion injury, a process that results in low or no flow as the result of microvascular occlusion secondary to endothelial cell injury.[10] Blockade of the αMβ2 receptor mitigates such injury in experimental models.[10,19] Platelets have also been implicated in mediating reperfusion injury; blockade of platelet glycoprotein IIb/IIIa receptors diminishes the reperfusion injury in the cerebral circulation in a mouse model.[20]

It is possible therefore that the blockade of the activated conformation of αMβ2 by abciximab may have beneficial effects on several phenomena that may improve the therapy of cardiovascular disease. It is important to emphasize, however, that it is unclear whether αMβ2 adopts the conformation that abciximab is capable of binding to during physiologic or pathologic processes, and flow cytometry studies of whole blood obtained from patients receiving abciximab therapy have not identified circulating abciximab-coated monocytes or granulocytes.[21] Because αMβ2 function is considered important in host resistance to infection, there is a theoretic risk that abciximab may increase the likelihood of the development of infection. To date, however, such a predisposition has not been reported.

The αVβ3 receptor

The αVβ3 receptor was originally designated the vitronectin receptor, but it also binds fibrinogen, von Willebrand factor, osteopontin, and other arginine-glycine-aspartic acid-containing ligands.[22] The β3 subunit is identical to glycoprotein IIIa and the αV subunit is highly homologous to the αIIb subunit.[22] αVβ3 is expressed at high density on osteoclasts and some tumors and at low density on endothelial cells, smooth muscle cells, and other cells.[22] Trace amounts of αVβ3 are present on platelets (approximately 100 αVβ3 receptors per platelet compared with 100,000 glycoprotein IIb/IIIa receptors).[4] Activation of endothelium during angiogenesis appears to result in increased αVβ3 expression.[23-26] Similarly, in animal models, smooth muscle cell αVβ3 receptor expression increases dramatically after vascular injury, including those smooth muscle cells in the neointima, for a period of 7 to 14 days.[27,28]

The αVβ3 receptor has been implicated in a variety of functions including bone resorption, tumor invasion and metastasis, and cell adhesion and spreading.[22,26,29] Some of the potential roles for the αVβ3 receptor with regard to thrombosis and vascular phenomena are discussed.

Activated platelets

αVβ3 receptors on activated platelets have been implicated in platelet adhesion to osteopontin, which is present in atherosclerotic plaque,[27] and in platelet-mediated facilitation of thrombin generation. Thus Bennett et al[30] demonstrated that antibody 7E3 can inhibit adhesion of activated platelets to osteopontin. Moliterno et al[31] demonstrated that abciximab treatment prolongs the activated coagulation times of patients undergoing heparin therapy, and we were able to simulate this phenomenon in vitro by adding abciximab to anticoagulated blood.[32] To further study this phenomenon, we analyzed the effect of 7E3 on thrombin generation in a reconstituted system. We found that murine 7E3 and abciximab could inhibit platelet-mediated thrombin generation induced by tissue factor.[33] Although much of the inhibitory effect of 7E3 on thrombin generation is ascribable to the blockade of

glycoprotein IIb/IIIa receptors, studies with other monoclonal antibodies and peptide inhibitors and studies of the platelets of patients with Glanzmann thrombasthenia suggested that some of the 7E3 effect is due to the inhibition of αVβ3.[33] Subsequent studies by Byzova and Plow[34] demonstrated that prothrombin could bind to glycoprotein IIb/IIIa and activated αVβ3,[35] providing additional mechanistic information on the roles of glycoprotein IIb/IIIa and αVβ3 in generation of thrombin.

Endothelial cells

αVβ3 receptors on endothelial cells have been implicated in tumor angiogenesis[24-26] and the adhesion of sickle cells to endothelial cells.[36] In preliminary studies, we have demonstrated that antibody 7E3 can inhibit tumor angiogenesis in a mouse model[37] and the adhesion of human sickle cells to the endothelium of platelet-activating factor–treated rat postcapillary venules.[38]

Smo th muscle cells

αVβ3 receptors on smooth muscle cells have been implicated in intimal hyperplasia after vascular injury, and this may potentially contribute to "restenosis."[27,28,39,40]

A number of animal model studies have demonstrated that αVβ3 blockade can prevent intimal hyperplasia after vascular injury.[27,40-44] Thus the data from the Evaluation of 7E3 for the Prevention of Ischemic Complications (EPIC study), indicating a reduction in clinical restenosis in patients treated with abciximab, generated considerable interest in a potential role of αVβ3 blockade by abciximab as contributing to the observed benefit.[45] Several clinical trials have not, however, confirmed an effect of abciximab on restenosis,[46,47] casting doubt on any potential role of αVβ3 blockade by abciximab. However, since the upregulation of αVβ3 receptors on smooth muscle cells persists for 1 to 2 weeks after vascular injury in animal models,[27,28] the current duration of treatment with abciximab (12 hours) is probably inadequate to fully test whether abciximab, operating through αVβ3 blockade, can diminish intimal hyperplasia. The increased use of stents as part of percutaneous coronary interventions makes it particularly important to define the role for αVβ3 in intimal hyperplasia, because the primary mechanism of stent restenosis involves intimal hyperplasia, with little or no contribution from elastic recoil or wound contraction.[27]

In this regard, recent data from the Evaluation of Platelet IIb/IIIa Inhibitor for Stenting (EPISTENT) trial have demonstrated an 18% reduction in target vessel vascularization at 6 months in all stented patients who received abciximab therapy (versus placebo), which by subgroup analysis appears to be primarily due to a 51% reduction in target vessel revascularization in diabetic patients with stents ($P = .02$) who received abciximab therapy (versus placebo).[48] These intriguing data raise the possibility that abciximab therapy may preferentially decrease stent-related intimal hyperplasia in diabetic patients, a group that has been reported to have abnormalities in both platelets and vascular function.[49] One needs to be very cautious, however, in ascribing any effects of abciximab therapy in decreasing intimal hyperplasia after stent placement to the blockade of αVβ3 by abciximab, because the blockade of platelet glycoprotein IIb/IIIa receptors by abciximab theoretically could be responsible for the observed effect through several different mechanisms, which include decreased mural thrombus formation, decreased release of growth factors from platelets (platelet-derived growth factor, adenosine diphosphate, serotonin) known to enhance smooth muscle cell–mediated intimal hyperplasia, and decreased platelet-mediated thrombin generation.[50]

Despite a multitude of proposed functions for αVβ3, there is no evidence that short-term blockade of αVβ3 by abciximab produces any adverse side effects. This is consistent with the similarities in hemorrhagic symptoms and lack of nonhemorrhagic abnormalities in individuals with Glanzmann thrombasthenia who are deficient in glycoprotein IIIa ([β3]; who lack both glycoprotein IIb/IIIa and αVβ3) compared with those individuals who are deficient in glycoprotein IIb (who lack glycoprotein IIb/IIIa but not αVβ3).[4]

Conclusions

The intriguing reactivities of abciximab with αMβ2 and αVβ3 receptors make it possible that some of the cardiovascular benefits observed with abciximab treatment reflect these effects. At present, however, there is no direct evidence that these reactivities play an important role. Similarly, at present, there are no data to indicate that these reactivities cause any adverse side effects. The current dosing of abciximab is probably not optimal for maintaining the blockade of αVβ3 receptors during the period of highest risk of αVβ3-mediated intimal hyperplasia, so a rigorous test of the potential effect of abciximab therapy on αVβ3-mediated intimal hyperplasia will probably require modifications of the current dosing regimen.

Animal model data support the potential novel utility of αVβ3 blockade in other disease states, such as sickle cell disease, and the inhibition of tumor angiogenesis, raising the possibility that abciximab may be useful for preventing sickle cell adhesion and inhibiting tumor angiogenesis. However, even if these preliminary animal data are confirmed in humans, it will remain to be established whether abciximab, with its additional glycoprotein IIb/IIIa blockade effect, will prove more beneficial than selective αVβ3 blockade.

I thank Suzanne Rivera for outstanding secretarial assistance.

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REVIEW

Potential Future Clinical Applications for the GPIIb/IIIa Antagonist, Abciximab in Thrombosis, Vascular and Oncological Indications

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Abciximab (ReoPro®) is a mouse-human chimeric monoclonal antibody Fab fragment of the parent murine monoclonal antibody 7E3, and was the first of these agents approved for use as adjunct therapy for the prevention of cardiac ischemic complications in patients undergoing percutaneous coronary intervention (PCI). Abciximab binds with high avidity to both the non-activated and activated form of the GPIIb/IIIa receptor of platelets, the major adhesion receptor involved in aggregation. Additional cardiovascular indications for abciximab are unstable angina, carotid stenting, ischemic stroke and peripheral vascular diseases. Abciximab also interacts with two other integrin receptors; the $\alpha_v\beta_3$ receptor, which is present in low numbers on platelets but in high density on activated endothelial and smooth muscle cells, and αMβ2 integrin which is present on activated leukocytes. Cell types that express integrins GPIIb/IIIa and $\alpha_v\beta_3$ such as platelets, endothelial and tumor cells have been implicated in angiogenesis, tumor growth and metastasis. Since abciximab interacts with high avidity to integrins GPIIb/IIIa and $\alpha_v\beta_3$, it is reasonable to assume that it may possess anti-angiogenic properties in angiogenesis-related diseases, as well as anti-metastastatic properties in case of disseminating tumors expressing the target integrin receptors. (Pathology Oncology Research Vol 6, No 3, 163–174, 2000)

Keywords: Abciximab, ReoPro®, cardiocascular disease, angiogenesis, tumor metastasis

Introduction

Platelet activation and subsequent platelet thrombus formation play a pivotal role in the pathophysiology of arterial thrombosis and subsequent acute coronary syndromes (ACS)[1,2] and have been strongly implicated in the development of noncardiac vascular diseases such as ischemic stroke,[3,4] carotid artery occlusion,[5] and peripheral vascular disease.[6] Platelet thrombus formation can be propagated by a number of complex, independent pathways and physiological stimuli.[1,7] Regardless of the mechanistic or chemical stimuli utilized, the end product of each of these pathways is the induction of a conformational change in the platelet glycoprotein (GP) IIb/IIIa receptor, which then allows the receptor to bind fibrinogen and other multivalent, adhesive proteins, ultimately resulting in platelet-platelet crosslinking and aggregate formation. GPIIb/IIIa antagonists have been developed to inhibit the interaction of fibrinogen and other ligands to the activated GPIIb/IIIa receptor, thereby inhibiting platelet aggregation and subsequent thrombus formation. GPIIb/IIIa antagonists are more versatile anti-platelet agents than those designed to inhibit one pathway of activation (e.g. heparin, ticlopidine, clopidogrel and aspirin) since they inhibit the final consequence of platelet activation – the interaction of fibrinogen with the activated GPIIb/IIIa receptor.[2] Validity of the concept of GPIIb/IIIa antagonism has been demonstrated in patients with symptomatic coronary artery disease undergoing percutaneous interventions.[8-12] In these studies, periprocedural administration of a parental GPIIb/IIIa antagonist markedly reduced the incidence of death or non-fatal myocardial infarction over the ensuing 30 days.

Abciximab (ReoPro®) is a mouse-human chimeric monoclonal antibody Fab fragment of the parent murine monoclonal antibody 7E3,[13] and was the first of these agents approved for use as adjunct therapy for the prevention of cardiac ischemic complications in patients undergoing

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percutaneous coronary intervention (PCI), or in unstable angina patients when PCI is planned within 24 hours. Abciximab binds with high avidity to both the non-activated and activated form of the GPIIb/IIIa receptor and the recommended dose induces 80% blockade of the GPIIb/IIIa receptor throughout the duration of treatment.[14-16] A number of large prospective clinical studies have established that abciximab affords long-term reduction (up to 3 years) in ischemic complications associated with PCI.[17,18] Additional cardiovascular indications for abciximab currently being explored are medical treatment for unstable angina, and combined therapy with reduced doses of the fibrinolytic Retavase®, in the setting of both acute myocardial infarction and facilitated PCI (see below). In addition, the utility of abciximab in non-cardiac vascular indications such as carotid stenting, ischemic stroke and peripheral vascular disease is also being explored.

During its development, it was reported that abciximab also interacts with two other integrin receptors (*Table 1*); the $\alpha_v\beta_3$ receptor (also known as the vitronectin receptor),[19] which is present in low numbers on platelets (100 copies per cell)[20] and in high density (500,000 copies per cell),[19] on activated endothelial and smooth muscle cells and αMβ2 integrin, also known as MAC-1, which is present on activated leukocytes.[21,22] Numerous *in vitro* and animal studies have associated $\alpha_v\beta_3$ and Mac-1 with a variety of pathophysiologic processes associated with acute coronary syndromes. Thus, it is postulated that some of the clinical benefits derived from abciximab in PCI patients could be correlated with cross-specificity with one or both of these receptors. Additionally, these receptors have been associated with non-cardiac disease processes, indicating that abciximab, or other compounds with equivalent specificity, may confer clinical benefit in other conditions where receptor activation is proposed. This article summarizes the scientific rationale and clinical evidence for the use of abciximab in additional cardiovascular and non cardiac vascular indications.

Potential Role for Abciximab as an Anti-Cancer Agent

The challenge for the oncology field today is to develop therapies that will combat advanced disseminated disease. Developing agents that will block the metastatic cascade and the growth of disseminated tumors are areas of research that are undergoing scrutiny from academic institutions and the pharmaceutical industry. One hopeful approach is anti-angiogenic therapy, where it is speculated that depleting the tumor of its blood supply may ultimately shrink the tumor and prolong patient survival. Cell types that express integrins GPIIb/IIIa and $\alpha_v\beta_3$ such as platelets, endothelial and tumor cells have been implicated in tumor growth, angiogenesis and metastasis. Since abciximab interacts with high avidity to integrins GPIIb/IIIa and $\alpha_v\beta_3$, it is reasonable to assume that it may possess anti-angiogenic properties. The following sections will provide a brief review of the importance of GPIIb/IIIa and $\alpha_v\beta_3$ integrins in tumor growth and metastasis, and the rationale for the development of abciximab as a therapeutic agent for certain cancers.

Role for $\alpha_v\beta_3$ Integrin in Tumor-Induced Angiogenesis

The outcome of solid tumor growth is closely associated with vascular density. Blockade of neo-vascularization can result in a significant decrease in solid tumor growth.[24,25] Tumor-secreted growth factors and inflammatory cells that infiltrate the tumor can initiate tumor-induced angiogenesis. Two such angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), stimulate endothelial expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which then results in new vessel formation. A variety of studies have indicated that blockade of $\alpha_v\beta_3$ function by antagonists suppresses angiogenesis and tumor growth in animal models.[26-28] One such antagonist LM609, a murine monoclonal IgG antibody that specifically blocks $\alpha_v\beta_3$, inhibits angiogenesis by pro-

Table 1. **Integrin specificity characteristics of abciximab**

Receptor	*Cell Expression*	*Receptor Density (molecules/cell)*	*Abciximab Affinity K_D*
$\alpha_{IIb}\beta_3$ (GPIIb/IIIa)	Platelets	≈80,000 (platelets)[1]	6.2 nM (platelets)[2]
$\alpha_v\beta_3$ (vitronectin receptor)	Platelets, fibroblasts, osteoclasts, PMN's, lymphocytes, tumors, endothelial and smooth muscle cells	≈100 (platelets)[3] ≈500,000 (cultured human endothelial cells [HUVECS])[4]	9.8 nM (HUVECS)[4]
$\alpha_M\beta_2$ (Mac-1; CD18/CD11b)	Activated lymphocyte[5,6]	200,000 (peripheral blood monocytes [PBM's])[6,7]	160 nM (PBM's)[6,7]

[1]Reference number (23). [2]Reference number (14). [3]Reference number (20). [4]Reference number (19). [5]Reference number (21). [6]Reference number (22). [7]Estimates obtained with the bivalent form of abciximab (7E3 IgG)

moting programmed cell death in activated, but not resting, endothelial cells via a P-53 dependent pathway.[29] In addition to integrins, proteinases may also be involved in angiogenesis by remodeling the sub-endothelial matrix, thus facilitating endothelial attachment and movement. Integrin $\alpha_v\beta_3$ can bind to matrix metalloproteinase-2 and inhibition of this interaction also inhibits angiogenesis in tumor model systems.[30] Immunohistological analysis indicated that expression of $\alpha_v\beta_3$ is preferentially enhanced in blood vessels of patients with human breast carcinoma.[31] These investigators also reported that integrin expression was significantly higher in tumors of patients with metastasis than patients without metastasis.

A variety of $\alpha_v\beta_3$ antagonists such as small molecular weight inhibitors, peptidomimetics, or monoclonal antibodies are in various stages of development as anti-cancer therapeutics. A selective peptidomimetic antagonist of $\alpha_v\beta_3$ SC-68448, inhibited endothelial cell proliferation and tumor growth in mice. In a mouse Matrigel model of angiogenesis, an $\alpha_v\beta_3$ small molecule antagonist SM 256, inhibited bFGF stimulated blood vessel formation.[27] A phase I clinical trial of the humanized version of LM609 (Vitaxin®) in patients with stage IV disease with breast, colon, lung, kidney and ovarian carcinoma has recently been completed with no reported major toxicity.[33] These findings collectively suggest that $\alpha_v\beta_3$ antagonists may be effective and well-tolerated anti-angiogenic agents.

Based on the available data supporting the role of $\alpha_v\beta_3$ in angiogenesis, abciximab was evaluated for anti-angiogenic activity. The parent antibody of abciximab, m7E3 IgG was compared to LM609 in a severe combined immuno-deficient (SCID) mouse human chimeric skin angiogenesis model.[34] In this system, $\alpha_v\beta_3$ negative human melanoma cells were injected into full-thickness human skin grafted onto SCID mice. The resulting tumors induced an angiogenic response that enhanced growth of tumor cells in an orthotopic microenvironment. Regular administration of 7E3 prevented or significantly inhibited growth of tumors, and this effect directly correlated with a reduction in the number of blood vessels supplying the tumors. Since 7E3, like LM609, does not crossreact with mouse integrins, its anti-angiogenic effect was attributed to blockade of human $\alpha_v\beta_3$ receptors in the vasculature of the human skin.

Role for Platelet GPIIb/IIIa Integrin in Tumor Growth and Metastasis

The rationale and evidence supporting the development of anti-platelet agents in metastasis has been reviewed in detail elsewhere.[35] Despite uncertainties as to how anti-platelet agents may function as anti-cancer therapeutics, some have postulated that these agents inhibit the adherence or trapping of cancer cells to capillary walls, exposing circulating tumor cells for a prolonged period of time to host anti-tumor entities. The involvement of platelets in experimental models of metastasis was recognized almost 30 years ago,[35] as an integral part of the microthrombus that is thought to be involved in the arrest of circulating tumor cells.

Certain tumor cells induce platelet aggregation (TCIPA) *in vitro*, which directly correlates with their metastatic potential.[36] It is hypothesized that TCIPA may be required during the hematogenous spread of tumor cells and the resulting aggregates induce endothelial cell retraction and facilitate tumor extravasation. This idea was supported by a finding that reconstituting thrombocytopenic mice with human platelets dramatically increased the lung colonization ability of tumor cells *in vivo*.[37] TCIPA can be blocked by mAbs directed to platelet GPIIb/IIIa integrin, 10E5 and AP-2[36,38-40] and abciximab (unpublished results, Trikha, M). The initial tumor cell-platelet bridging event requires β_3 integrins and this results in a rapid induction of platelet aggregation. Furthermore, blockade of human platelet GPIIb/IIIa by 10E5 blocked the increase in lung colonization of tumor cells.[37] These results suggested that platelets use GPIIb/IIIa integrin to interact with circulating tumor cells and blockade of this receptor could prevent tumor cell arrest and/or extravasation.

Recently, Amirkhosravi et al. demonstrated that murine tumor cells injected intravenously into nude rats rapidly induced thrombocytopenia.[41] The murine $F(ab')_2$ version of abciximab, 7E3 $F(ab')_2$ that crossreacts with rat, but not murine, GPIIb/IIIa and $\alpha_v\beta_3$ prevented tumor cell-induced thrombocytopenia. The functional consequence of blocking tumor cell-induced thrombocytopenia was a near complete eradication of experimental metastasis. The authors speculated that when tumor cells are shed into the circulation, they rapidly recruit platelets to form tumor cell-platelet aggregates which results in a transient decrease in circulating platelet count. These aggregates help tumor cells survive the hostile environment and facilitate in their arrest at distant sites. These studies in conjunction with results obtained from 10E5 experiments suggest that abciximab could block hematogenous metastasis.

In addition to facilitating hematogenous metastasis, platelets may also participate in angiogenesis and growth of primary and disseminated tumors. Pinedo and Folkman have postulated that a true anti-angiogenic therapy must target platelets.[42] Platelets contain one of the largest stores of angiogenic and mitogenic factors, and with a circulating half life of ~5-7 days,[43] they could provide tumors with a continuous supply of growth factors. Tumor vasculature is leaky and extravasated fibrin(ogen) that is deposited on the tumor surface can provide an ideal substrate for platelet binding. Platelet granules contain a variety of factors such as VEGF, PDGF, TGF-β, and fibrinogen, and these modulators are immediately secreted after platelet

activation. Abciximab can block platelet aggregation and adhesion to fibrin(ogen), and it also inhibits platelet degranulation. By blocking granule release, abciximab inhibits secretion of serotonin, TGF-β, PDGF AB[44] and VEGF.[41,45,46] Most of these factors have been implicated in various steps of tumor progression and metastasis. VEGF is one such angiogenic factor that is stored in large amounts in circulating platelets. Abciximab inhibits ADP-stimulated platelet secretion of VEGF.[46] In addition, tumor cells induce platelets to secrete VEGF and this secretion is also blocked by abciximab.[41] Blockade of VEGF secretion by abciximab is due to its ability to inhibit both platelet aggregation and tumor cell-platelet binding that is mediated by $\alpha_v\beta_3$ and platelet GPIIb/IIIa. It is tempting to speculate that when administered to patients with cancer, abciximab could directly block $\alpha_v\beta_3$ and GPIIb/IIIa function and indirectly block VEGF function. This multi-receptor binding of abciximab may distinguish it from other anti-angiogenic antagonists that are unable to inhibit platelet GPIIb/IIIa. However, the safety of this dual effect of abciximab remains to be defined.

Role of Tumor β3 Integrins in Tumor Growth and Metastasis

In addition to participating in host cell mediated tumor growth, angiogenesis and metastasis, β_3 integrins are also upregulated in certain tumors. A large body of literature indicates that β_3 integrins play a critical role in mediating human melanoma cell adhesion, spreading, invasion, and tumor cell survival *in vitro* and in animal tumor models.[36] The clinical significance of β_3 integrin expression was suggested in a prospective study that examined the expression of this integrin in patients who were followed for a mean of 98 months post-diagnosis with intermediate thickness malignant melanoma.[47,48] This study concluded that tumors in 64% of the patients expressed β_3 integrin, with greater mortality in patients with β_3 positive melanomas when compared to those with β_3 negative tumors (45% vs. 8%). Presence of β_3 integrin was also associated with subsequent lung metastasis. In an earlier study, Hsu et al., demonstrated that adenoviral gene transfer of the β_3 integrin subunit into non-tumorigenic radial growth phase primary human melanoma cells converted the cells into tumorigenic and invasive vertical growth phase primary melanoma.[49] Collectively, these observations suggest that blockade of β_3 integrin function in human melanoma may suppress tumor growth and metastasis.

An important role of tumor expressed $\alpha_v\beta_3$ was demonstrated when function blocking mAb LM609 induced apoptosis of human melanoma cell growth in a collagen gel.[50] Native type I collagen does not bind $\alpha_v\beta_3$ integrin, but tumor cell secreted matrix metalloproteinases can degrade type I collagen to expose cryptic sites that recognize $\alpha_v\beta_3$. LM609 inhibits melanoma cell binding to these exposed sites in denatured collagen thereby promoting apoptosis. Cheresh and colleagues demonstrated that M21-L cells that do not express $\alpha_v\beta_3$ are significantly less tumorigenic in nude mice when compared to the parental M21 cells that are positive for $\alpha_v\beta_3$ integrin.[51] Subsequently, they demonstrated that repeat administration of LM609 inhibits growth of M21 ($\alpha_v\beta_3$ positive) cells in mice. Since LM609 does not recognize mouse $\alpha_v\beta_3$, these data suggest that $\alpha_v\beta_3$-expressed in human melanoma may contribute to tumor growth independent of its role in angiogenesis.[52]

In addition to the important role of $\alpha_v\beta_3$ integrin in tumor growth and metastasis, a subpopulation of tumors abnormally express the platelet GPIIb/IIIa integrin. Puerschel et al., evaluated human melanoma specimens for expression of GPIIb and GPIIIa subunits on cells from patients with metastatic and non-metastatic malignant melanoma over a 6 year period.[53] They observed that the GPIIb subunit was present exclusively on metastatic melanoma cells, but not on non-metastatic melanomas or benign melanocytes. As expected, GPIIIa (also known as β_3) was heterogeneously expressed in both primary and metastatic melanoma. The absence of contaminating platelets in the tumor specimens was ruled out by staining with the platelet specific GPIb antibody. Others studies corroborating these findings reported that in an experimental model of metastatic melanoma, expression of GPIIb/IIIa directly correlated with metastatic potential.[36,38,39] Further, GPIIb/IIIa expression can be detected in some solid tumor cell lines and function blocking mAbs directed to GPIIb/IIIa block tumor cell adhesion, metastasis and invasion.[54-58] Taken together, these findings suggest that targeting GPIIb/IIIa and $\alpha_v\beta_3$ may be a more effective therapy for certain cancers than targeting either integrin alone.

Considerations

An important message from the findings reviewed above is that combined blockade of integrins GPIIb/IIIa and $\alpha_v\beta_3$ may be more effective than a therapy that targets only single receptors. Abciximab inhibits GPIIb/IIIa and $\alpha_v\beta_3$ with equivalent affinity and has been administered to over a million patients with atherosclerotic disease with minimal complications. However, the safety of chronic administration of abciximab in patients with advanced cancer (ic bleeding, formation of reston bock an immune response to abciximab) as well as the ability to administer an effective concentration of the drog at the tumor site neds to be evaluated. The pharmacodynamics of abciximab within the tumor microenvironment must be closely examined to ensure adequate drug delivery. Currently, there are few effective therapies available for patients with advanced cancer. As described above, *in*

vivo tumors interact with a variety of host cells such as platelets and endothelial cells, and these interactions help them to grow and metastasize. The ability of abciximab to block many such interactions suggests a novel approach with the potential to inhibit tumor progression.

Sickle Cell Crisis

Sickle cell disease is an autosomal dominant genetic disorder characterized by red cells that transform into a sickle cell shape upon deoxygenation.[59] The genetic defect is a point mutation which substitutes a valine for glutamic acid in the sixth position of the β-globin chain and results in the abnormal polymerization of sickle hemoglobin under hypoxic conditions.[60] The pathophysiology of the disease is related to the intracellular polymerization of sickle cell hemoglobin and the abnormal interaction of sickle erythrocytes with the microvascular endothelium.[61] The clinical manifestations of sickle cell disease are highly diverse, but are all ultimately linked to hemolytic anemia and recurring episodes of painful vascular occlusion. Such occlusions are associated with multiple organ damage and increased susceptibility to infection, primarily with polysaccharide-encapsulated organisms due to splenic infarction. The vascular occlusion can involve every organ of the body.

The distribution of sickle cell disease parallels that of falciparum malaria since people who carry the sickle cell trait and are infected with plasmodium falciparum have a selective advantage over those not carrying the gene.[62] Due to the selective advantage of sickle cell over the normal gene, the frequency of the trait is increased in areas where malaria is endemic. There are approximately 2.5 million people in the US and 30 million people in the world who have sickle cell trait.[63] The incidence of sickle cell trait in the US is 1 in approximately 600 newborns, with a significant incidence among the African American population (8%).[64]

The pattern of illness and disease severity varies considerably among individuals homozygous for hemoglobin S. However, the syndrome is associated with significant morbidity and mortality that is primarily mediated by the vaso-occlusion of the microvasculature. The underlying pathophysiology of these vaso-occlusive episodes is complex and may involve adhesion receptor mediated interactions of sickle cell red blood cells (SS RBC's), other cellular constituents (e.g. platelets, leukocytes) and the endothelial cells lining the vascular bed.[65] These adhesion receptors are up-regulated by inflammatory mediators that are produced during infection or inflammation.[66]

A number of *in vitro* studies have implicated several adhesion receptors (e.g. α4β1 and CD36 on SS RBC's and VCAM-1 and $\alpha_v\beta_3$ on activated endothelial cells)[66-69] and adhesive proteins (von Willebrand factor [vWf], thrombospondin, fibrinogen)[70-72] in mediating the association of SS RBC's with endothelial cells, yet the significance of one particular receptor or ligand in the development of microvascular occlusion has until recently, not been clearly elucidated. Kaul and colleagues,[73] using an *ex vivo* platelet activating factor (PAF) activated rat mesocecal microvascularization model, demonstrated that a bivalent form of abciximab (7E3 F(ab')$_2$) and an $\alpha_v\beta_3$-specific antibody LM609 appreciably reduced adhesion of human sickle cells to postcapillary venules. In contrast, a GPIIb/IIIa-specific antibody 10E5 had no effect on the hemodynamics of SS RBC's in PAF-treated-vessels. PAF, a potent inflammatory agent that is elevated in the plasma of sickle cell patients,[74] and increases endothelial vWf expression,[75] was used to stimulate the interaction of SS RBC's with the post-capillary venules. This and/or other pro-inflammatory agents with similar physiological effects that are produced during infection may play a pivotal role in the development of sickle cell-mediated vascular occlusion. These data, as well as numerous *in vitro* studies, support the role of $\alpha_v\beta_3$ in mediating SS-RBC interactions with endothelial cells lining postcapillary venules. It should be noted that certain limitations relating to the experimental design need to be addressed. This experimental model consisted of isolated cells in a plasma-free medium and does not reflect the complex hemodynamics that occur in the microcirculation during an inflammatory episode. For instance, subjects with sickle cell disease have increased numbers of circulating platelet-erythrocyte aggregates and elevated levels of platelet activation, *in vivo*.[76,77] Adhesive glycoproteins (e.g. thrombospondin) released from activated platelets may exacerbate microvascular occlusion by promoting sickle cell adhesion to the endothelium. Thus, the current *in vitro* and *ex vivo* data provides the rationale for dual GPIIb/IIIa and $\alpha_v\beta_3$ receptor blockade as a therapeutic approach to prevent sickle cell disease-related vascular occlusion.

Stroke

Stroke is an important clinical disorder associated with significant morbidity, mortality and economic impact. It is the second leading cause of adult mortality and the leading cause of serious disability in older individuals.[78,79] In the United States, there are an estimated 500,000 to 700,000 new cases of stroke (85% of which are ischemic), and 150,000 deaths attributed to the disease each year.[80] Twenty (20%) of stroke victims die within the first month after the episode.[81] For those who survive for 6 months, approximately 15% require institutional care and 30–40% are dependent in their daily living.[82] The combined incidence of acute ischemic stroke in the United States and Europe is in excess of 1.2 million cases per year.[83] The aggregate lifetime cost of ischemic stroke occurring within a single

year in the United States has been estimated at $29 billion, and the average lifetime cost for a stroke victim from diagnosis to death is approximately $90,000.[84] These numbers are expected to rise as the median survival age of humans increases.[85]

The only approved reperfusion treatment for ischemic stroke is alteplase (Activase®), a recombinant tissue plasminogen activator (rt-PA).[86,87] However, <5% of stroke patients are candidates for this therapy, primarily due to its narrow therapeutic window (institution of therapy less than 3 hrs after symptom onset), and the associated increased risk of intracranial hemorrhage. The pivotal rt-PA trial that was conducted by the National Institute of Neurological Disorders and Stroke (NINDS)[86] resulted in 120 fewer deaths or disabled patients per 1000 patients treated, or a 12% overall benefit. However, the incidence of symptomatic intracranial hemorrhage increased 10-fold, from 0.6% in placebo patients to 6.4% in subjects that received rt-PA. Attempts at demonstrating benefits of initiation of fibrinolytic therapy up to six hours after symptom onset have been discouraging.[86-91] Thus, the narrow treatment window and significant intracranial hemorrhage risk associated with the use of rt-PA necessitates the development of safer and more efficacious reperfusion treatments that demonstrate efficacy if the therapy is initiated beyond three hours after symptom onset.

The rationale for GPIIb/IIIa antagonist therapy in ischemic stroke is derived from clinical reports with other anti-platelet agents as well as studies of abciximab in acute myocardial infarction patients. The results of two recent large, randomized trials, the International Stroke Trial[3] and the Chinese Acute Stroke study[4] suggest that platelets play an important part in the pathophysiology of acute ischemic stroke. The combined analyses of these studies revealed that administration of aspirin within 48 hours after onset of symptoms resulted in 13 fewer deaths or disabled patients per 1000 patients treated. It is currently unclear whether the therapeutic benefits of aspirin are attributed to its anti-platelet or anti-inflammatory actions, or a combination of both. However, additional support for the use of anti-platelet therapy in ischemic stoke is the demonstration that platelet ADP receptor antagonists, ticlopidine and its second generation counterpart, clopidogrel are efficacious in secondary prevention of stroke.[92] Thus, it is feasible that a more potent anti-platelet agent, such as a GPIIb/IIIa antagonist may confer more optimal clinical benefit over partial antagonists.

The evidence indicating that abciximab may be a safer reperfusion agent than rt-PA in ischemic stroke is derived from clinical studies of the agent in patients with acute coronary syndromes. The intracranial hemorrhage rate from the coronary intervention trials with abciximab is very favorable – 0.1% or 1 per 1000 patients.[9-12] Additionally, angiographic studies in acute myocardial infarction (TIMI14a and the GUSTO IV pilot trials) demonstrated that the combination of abciximab, aspirin and weight adjusted heparin reperfused occluded coronary vessels.[93,94] A composite analysis from both studies revealed abciximab resulted in a 45% patency rate and was comparable to historical reperfusion rates achieved with streptokinase. The mechanism(s) by which abciximab re-establishes flow in occluded vessels is unknown, but may be related to its ability to compete with and dissociate platelet bound fibrinogen on the activated platelet GPIIb/IIIa receptor, and by preventing platelet deposition on existing thrombi, thus allowing endogenous fibrinolysis to proceed unopposed. Abciximab has the potential to enhance thrombolysis by inhibiting release and deposition of platelet-derived plasminogen inhibitors at the site of thrombus.[44] Additionally, abciximab may further destabilize clot structure by impeding clot retraction[95] and factor XIIIa crosslinking of fibrin and plasminogen activator inhibition-1 (PAI-1) to the platelet mesh.[96]

A phase I, placebo-controlled, dose escalation study of abciximab in ischemic stroke was recently completed, and the results are promising.[97] The main objective of the trial was to determine the safety of abciximab in the setting of acute ischemic stroke. Patients presenting within 24 hrs of their stroke onset were randomized to receive, (in a 3 to 1 ratio), a single, escalating dose of abciximab or placebo. Patients were stratified according to the time of stroke onset and stroke severity. The highest dose of abciximab administered was equivalent to the dose recommended for patients undergoing PCI (0.25 mg/kg bolus and a 0.125 μg/kg/min infusion for 12 hrs). No symptomatic ICH bleeds were observed in either the placebo or abciximab groups during the 3 month follow-up period. At 3 months, there was a trend towards improved functional status among abciximab patients, compared to the placebo group. Based on the results of this study, a larger, double-blind, placebo-controlled study is currently being designed to assess the efficacy of abciximab in patients with ischemic stroke. The primary objective of the study is to assess patient disability at 3 months, using a modified Rankin Scale.

Chastain et al.,[98] evaluated the effect of abciximab in patients undergoing cerebral vascular stenting. Abciximab was used either prophylactically or as emergency bail-out following distal atherosclerotic debris embolization. The mean percent vessel stenosis prior to PCI was 75.9 ± 17.4% and was reduced to 6.2 ± 9.1% after PCI. There were no thromboembolic complications. Hemorrhagic complications occurred in two patients, one resulting in death from an occult berry aneurysm. With the exception of one patient who experienced a minor stroke, each of the patients in whom abciximab was used for bailout following distal debris embolism recovered completely.

Table 2. **Overview of TIMI14 and SPEED Trials**

	*TIMI 14**	*Speed/Gusto IV AMI Pilot***
Criteria	Age 18 to 75 Symptoms ≤ 12 hrs	≥ 18 years old Symptoms ≤ 6 hrs
Control Arm(s)	Accelerated t-PA or 10 U + 10 U Reteplase	Standard Reteplase (10 U + 10 U)
Combination Therapies Studied	t-PA, SK, and Reteplase Abciximab*** Heparin 60 U/kg and 30 U/kg	Reteplase + Abciximab*** Heparin 60 U/kg and 40 U/kg
Primary Endpoint(s)	TIMI grade 3 flow at 90 minutes	TIMI grade 3 flow at 60 and 90 minutes
PCI	Rescue only	"Encouraged" -Facilitated PCI

*Ref 94. **Ref 93. ***bolus 0.25 mg/kg; infusion 0.125 μg/kg/min H 12 hrs

Management of AMI with Fibrinolytics and GP IIb/IIIa Receptor Inhibitors

We are now entering a new age in the treatment of acute myocardial infarction (AMI). The past decade has seen great emphasis on the use of fibrinolytic agents in the treatment of AMI. Early success with first generation fibrinolytic agents (e.g., streptokinase) lead to the development of second (e.g., alteplase) and third generation fibrinolytic agents (e.g., reteplase, tenecteplase, lanoteplase) with the hope that altering certain characteristics of the plasminogen activator (e.g., fibrin affinity, fibrin specificity, PAI-1 inhibition, etc) would lead to improvements in TIMI 3 (normal coronary) flow rates and improved survival benefit. Unfortunately, while the objective of achieving bolus administration was achieved, these efforts were not successful in increasing survival. Three large (approximately 16,000 patients each) clinical trials[99-101] all failed to demonstrate a survival benefit of one agent over another.

Mechanistic evaluation now suggests an explanation for these clinical findings. Fibrinolytics act only on the fibrin mesh that aggregates platelets, red cells, and thrombin together. They do not address the ongoing stimulation of platelets, the release of clot-bound thrombin during fibrinolysis, new thrombin generation, or the initiation of the thrombotic cascade that proceeds on the platelet surface. Not only are activated platelets exposed following fibrinolysis, but released thrombin is a potent activator of platelets and studies have demonstrated a paradoxical activation of platelets following fibrinolysis. Furthermore, fibrinolysis may cause athero-embolization, a process that can activate platelets and lead to obstruction of the microvasculature in the myocardium. Thus, to optimize vessel patency, minimize the possibility of reocclusion (which occurs in 15% to 20% of cases following fibrinolysis), and protect/improve flow into the microcirculation, platelet inhibition during fibrinolysis would be desirable. Recently completed Phase II clinical trials provide evidence that the addition of the platelet inhibitor abciximab to the fibrinolytic agents reteplase or alteplase provides earlier and more complete reperfusion, reduction or elimination of cyclic flow variation and reocclusion, and improvements in microvascular obstruction.

Early trial data supported the concept of the benefits of combining platelet blockade with fibrinolysis. The ISIS-II trial demonstrated an additive effect when the platelet inhibitor aspirin was combined with streptokinase. When used in combination, the two agents provided a 42% reduction in mortality versus placebo.[102] However, as previously noted, drugs targeted at blocking only one pathway of platelet activation achieve only partial platelet inhibition because of the numerous, redundant pathways involved in platelet function. The more potent GPIIb/IIIa receptor inhibitors prevent aggregation regardless of the pathway of activation. In fact, several studies were undertaken in the early 1990's to examine the benefit of GP IIb/IIIa blockade with of fibrinolytics.[103-105] While successful in demonstrating more rapid and complete clot lysis, these studies suffered from increased bleeding rates presumably due to the use of full dose lytic, high dose heparin, and non-optimal access site management.

The safety and efficacy of combination therapy using full dose abciximab with reduced-dose lytic agent (reteplase, alteplase, or streptokinase) has recently been evaluated in two Phase II clinical trials, TIMI 14, and the pilot phase of the GUSTO IV AMI trial, SPEED *(Table 2;* ref 93,94). In the **S**trategies for **P**atency **E**nhancement in the **E**mergency **D**epartment (SPEED) trial, the pilot for the GUSTO-IV AMI study, abciximab administration alone was compared with combination therapy consisting of abciximab and various doses of reteplase.[93] The combination of full dose abciximab and half-dose (5 units + 5 units) reteplase resulted in accelerated optimal TIMI 3 flow rates, compared to full-dose lytic alone control *(Figure 1).* Reduced dose heparin, while marginally safer in terms of bleeding rates, led to reduced TIMI-3 flow rates. Control arms included abciximab for the dose finding phase of the trial (demonstrating the dethrombosis effect

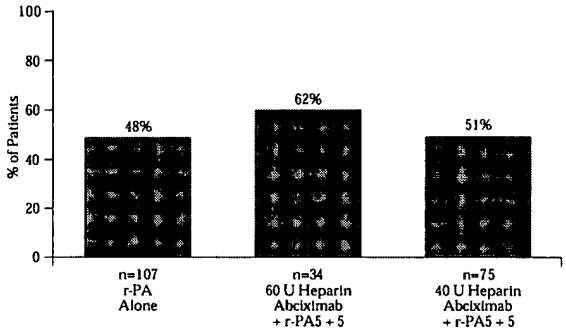

Figure 1. Angiographic core laboratory TIMI 3 flow rates at 60 min following r-PA alone (10 U + 10U), abciximab (0.25 mg/kg bolus and 0.125 μg/kg/min infusion for 12 hours) and combined lower dose r-PA and abciximab with variable heparin dosing. A significant trend towards increased TIMI 3 flow was observed with combined abciximab and half-dose r-PA. (Adapted with permission from Ohman et al., ref 93).

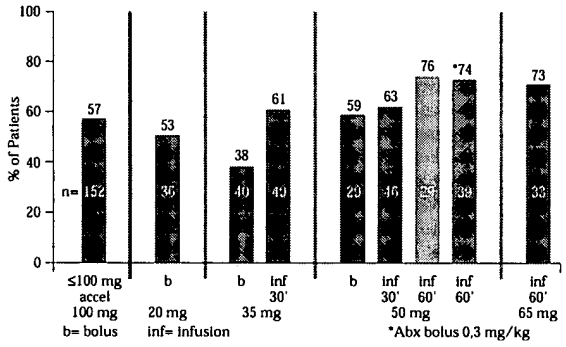

Figure 2. Angiographic core laboratory TIMI 3 flow from the dose-finding phase of the TIMI 14 trial. Variable flow rates are achieved between the different study agent protocols. A trend towards increased TIMI 3 flow at 90 min in patients receiving standard dose of abciximab and 50 mg t-PA was observed, compared to 100 mg t-PA alone. (Adapted with permission from Antman et al., ref 94).

of abciximab alone) and standard reteplase for the dose confirmation phase of the trial.

In TIMI-14 the combination of full-dose abciximab and reduced dose lytics were tested using safety and TIMI-3 flow at 90 minutes as the primary endpoints. A variety of doses of alteplase lead to the interesting observation that bolus administration was not as effective as bolus plus infusion. Optimal TIMI-3 flow rates were observed with a 15 mg bolus followed by a 35 mg infusion over 60 minutes (*Figure 2*).

In the reteplase phase, optimal TIMI-3 flow rates at 90 minutes were observed with half dose reteplase given as two 5 U boluses 30 minutes apart. Unusually high TIMI-3 flow rates were observed in the control (lytic alone) arm of this trial and especially in the reteplase control arm; this was found to be due to a disproportionate number of non-anterior myocardial infarctions as well as an earlier time to treatment in this control arm of the trial. Given the wide confidence intervals (due to the limited numbers of patients in each arm), these and other Phase II combination therapy data are most objectively viewed in terms of the trends observed with different lytic doses. The optimal doses of lytic (reteplase 5 U + 5 U) and heparin (60 U/kg bolus followed by 7 U/kg/hr) with full dose abciximab are being tested in the 16,600 patient Phase III GUSTO IV AMI trial which is presently underway to test the key clinical benefit (ie, mortality at 30 days) of combination therapy.

Facilitated Percutaneous Coronary Intervention

Percutaneous coronary intervention (PCI) appears to be effective in not only restoring perfusion but in resolving the underlying stenosis of the infarct-related artery. In a post-hoc analysis of the GUSTO III trial, a subset of patients underwent early angioplasty after failed fibrinolysis for whom complete data are available.[106] Death was significantly reduced in the patients who received abciximab at the time of their intervention (9.7% reduced to 3.6%, p = 0.042). At 30 days, the composite end point of death, stroke, or reinfarction occurred in 7.3% of patients who received reteplase and abciximab and 21.4% of patients who received alteplase and abciximab ($P = 0.08$; *Figure 3).*

These data suggest but do not prove that platelet inhibition, especially with GPIIb/IIIa antagonists, administered within 12 to 24 hours of thrombolysis may be advantageous, especially when used in conjunction with reteplase during rescue PCI.

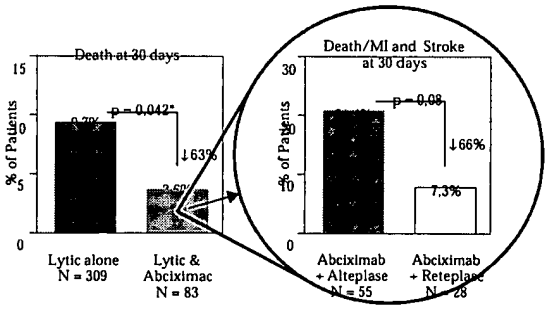

Figure 3. GUSTO III substudy data of survival benefits in patients receiving emergent abciximab therapy following thrombolytic therapy. A significant reduction in 30 day mortality was observed in patients receiving combined thrombolytic therapy. A significant trend within this small subgroup to even greater improvement was observed in patients receiving r-PA with regard to death, myocardial infarction and stroke. (Adapted with permission from Miller et al., ref 106).

Peripheral Vascular Disease

The use of abciximab in peripheral vascular disease, including intracranial interventions, carotid stenting, renal stenting, and peripheral arterial obstructive disease remains anecdotal with only case reports and small series but no controlled studies reported at this time. Abciximab has been used under two distinct but related circumstances. In the first, abciximab is used as an adjunct to lytic agents to enhance lysis of clots in extracardiac vessels.

In the second, abciximab is used as an adjunct to stenting with the goal of decreasing acute ischemic complications and possibly reducing restenosis, with the expectation of improved clinical outcomes in this difficult to treat patient population.

The rationale for the use of abciximab in these settings is clear. The underlying pathophysiology in peripheral vascular, renal, & carotid/intracranial vessel disease is atherosclerosis. As in the coronary vasculature, atherosclerotic plaques either rupture spontaneously or iatrogenically during peripheral interventions, activating both platelets and the coagulation cascade. Vascular interventions (including stenting) are a potent stimulus for platelet activation and aggregation, regardless of the location of the vessel. Finally, the incidence of diabetes is high (35–50%) in patients with peripheral vascular disease. Given the especially robust benefit of abciximab in reducing target vessel revascularlization in diabetic patients undergoing coronary stent placement in the EPISTENT trial,[106] a similar robust benefit would be obtained from the adjunctive use of abciximab in extracardiac vascular interventions in these patients.

While the administration of abciximab in extracardiac vascular disease has not yet been formally evaluated for safety and efficacy, its use has been reported in small case series in the medical literature and it is presently being studied in a variety of controlled clinical trials and registries.

Summary

As the foregoing discussion suggests, the adjunctive use of abciximab in percutaneous intervention appears in initial reports to be safe and effective for the treatment of extracardiac vascular disease. However, the details of the safety profile and utility of abciximab in extracardiac percutaneous interventions remain undefined. Additional data on the efficacy and safety of abcixmab use in these disease states awaits randomized clinical trials that are currently either in progress or in the design phase. Should these benefits be recognized, the use of abciximab will likely provide a major benefit to the care of patients with extracardiac vascular disease.

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Platelets and Cancer: Implications for Antiangiogenic Therapy

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ABSTRACT

Thromboembolism is one of the most common causes of death in cancer patients. Among the most frequent thrombotic complications in patients with cancer are disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, and thrombocytosis. Clearly, these complications arise as tumor cells interact with almost all components of the hemostatic system including platelets. Platelets participate in tumor progression by contributing to the metastatic cascade, protecting tumor cells from immune surveillance, regulating tumor cell invasion, and angiogenesis. Platelets contain one of the largest stores of angiogenic and mitogenic factors and the tumor vasculature is leaky, which allows platelets to come in contact with the tumor and deposit multiple angiogenic factors including vascular endothelial growth factor (VEGF) and thrombin to tumor cells, which in turn contributes to tumor progression. This article reviews the recent literature on how platelets contribute to tumor growth, angiogenesis, and metastasis.

KEYWORDS: Platelets, cancer, metastasis, angiogenesis, integrins

Objectives: Upon completion of this article, the reader should be able to (1) list how platelets contribute to cancer growth and angiogenesis, and (2) understand the rationale for using antiplatelet drugs in the treatment of cancer patients.
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The first association between hypercoagulability and malignancy dates back to the observations made by Trousseau in 1865, yet today thromboembolism is one the most common causes of death in cancer patients.[1] Several factors contribute to the increased risk of thromboembolic events in cancer patients. The most frequent thromboembolic complications in patients with malignant disease are deep vein thrombosis, disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, and thrombocytosis. Tumors possess both procoagulant and anticoagulant activity and are capable of interacting with all components of the hemostatic system including platelets. Although it is well accepted that platelets play an important role during hematogenous metastasis, the role of platelets in tumor growth and angiogenesis has been considerably less well investigated. The focus of this article is to review the recent literature on how platelets

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contribute to tumor growth, angiogenesis, and the metastatic cascade.

PLATELETS ENHANCE TUMOR CELL METASTASIS

The rationale and evidence supporting the development of anticoagulants and antiplatelet agents in metastasis has been reviewed in detail elsewhere.[2] Despite uncertainties as to how platelet antagonists may function as anticancer therapeutics, it is well accepted that these agents inhibit the adherence or trapping of cancer cells to capillary walls so that tumor cells remain in the systemic circulation and are exposed for a prolonged period to a hostile environment that can lead to the elimination of the tumor cells. The requirement of platelets in experimental models of metastasis was recognized almost 30 years ago.[2,3] Platelets are an integral part of the microthrombus that is thought to promote the arrest and lodgment of circulating tumor cells.

Certain tumor cells induce platelet aggregation (TCIPA), and this characteristic correlates directly with their metastatic potential.[3] TCIPA can be completely blocked by monoclonal antibodies (mAbs) directed to platelet glycoprotein (Gp) IIb/IIIa integrin, 10E5 and AP-2.[3-6] Abciximab is a chimeric Fab fragment of 7E3 that is currently used in patients undergoing percutaneous coronary intervention.[7-10] This mAb fragment inhibits platelet GpIIb/IIIa and αvβ3 integrin function and also inhibits TCIPA (M. Trikha, unpublished results, 2000). The initial tumor cell–platelet bridging event requires β3 integrins and secretion of tissue factor, and this results in a rapid induction of platelet aggregation. It is hypothesized that TCIPA may be required during the hematogenous spread of tumor cells and that tumor cell–platelet aggregates lodge in the microcirculation, induce endothelial cell retraction, and facilitate tumor cell extravasation. This idea was supported by a finding that tumor cell arrest under flow conditions required interaction of the tumor cells with platelets.[11] In this study platelet GpIIb/IIIa was postulated to bridge with tumor cell αvβ3, allowing the tumor cell–platelet aggregate to adhere to the vascular endothelium under physiologic shear conditions. In addition, the investigators found that primary metastatic cells from a patient with advanced breast cancer were highly adhesive for platelets.[11] Another study showed that reconstituting thrombocytopenic mice with human platelets dramatically increased the lung colonization ability of tumor cells in vivo.[12] Furthermore, blockade of human platelet GpIIb/IIIa integrin by mAb 10E5 blocked lung colonization of tumor cells.[12] These results suggested that platelets use GpIIb/IIIa integrin to interact with circulating tumor cells and that blockade of this receptor could prevent tumor cell extravasation.

TCIPA in some instances is caused by ADP, a platelet agonist, that is secreted by the platelets themselves after they have come in contact with tumor cells.[13] In other instances, platelets are activated by thrombin generation, possibly due to the expression of tissue factor, known to be expressed by a variety of tumor cells.[14-16] Tissue factor is a potent activator of thrombin generation. Thrombin, in addition to being a central player in thrombus formation, is the most potent platelet agonist and it also activates tumor cells.[17,18] Interestingly, platelets not only are activated by thrombin generation but also play a role in initiating thrombin generation. The platelet GpIIb/IIIa receptor is classically known to mediate platelet aggregation, but a recent study has shown that GpIIb/IIIa also mediates tissue factor–induced coagulation.[19] In this study, abciximab, and to a lesser extent eptifibatide, reduced thrombin generation and delayed clot formation. These results suggest that platelet blockade may not only provide antiadhesive benefit by preventing tumor cell–platelet interaction but also provide anticoagulant activity by preventing thrombin generation stimulated by tumor-derived tissue factor.

Certain tumor cells in circulation rapidly recruit platelets, thereby causing a transient thrombocytopenic effect. Amirkhosravi et al[20] have demonstrated that murine tumor cells injected intravenously into nude rats rapidly induced thrombocytopenia. The murine $F(ab')_2$ version of abciximab, 7E3 $F(ab')_2$, that cross-reacts with rat but not murine GpIIb/IIIa and αvβ3 prevented tumor cell–induced thrombocytopenia. The functional consequence of blocking tumor cell–induced thrombocytopenia was almost complete eradication of experimental metastasis. The authors speculated that when tumors are shed into the circulation, they rapidly recruit platelets to form tumor cell–platelet aggregates, which results in a transient decrease in circulating platelet count. These aggregates help tumor cells survive the hostile environment and facilitate in lodging at distant sites. These studies, in conjunction with results obtained from 10E5 experiments, suggest that inhibition of platelet GpIIb/IIIa prevents hematogenous metastasis.

PLATELETS REGULATE TUMOR ANGIOGENESIS

In addition to facilitating hematogenous metastasis, platelets may participate in the growth and angiogenesis of primary and disseminated tumors. Pinedo et al[21] have postulated that a true antiangiogenic therapy must target platelets. Platelets are extremely numerous (2×10^8/mL of blood) and contain one of the largest stores of angiogenic and mitogenic factors, and with a relatively short circulating half-life of ~5–7 days[22] they could provide tumors with a continuous supply of angiogenic factors. Tumor vasculature is leaky, and ex-

travasated fibrin(ogen) that is deposited on the tumor surface can provide an ideal substrate for platelet binding. Platelet granules contain a variety of factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor β(TGF-β), interleukin-6 (IL-6), thrombin, and fibrinogen, and these modulators are secreted immediately after platelet activation.[20,23-25] Most of these factors have been implicated in various steps of tumor progression and metastasis. VEGF is one such angiogenic factor that is stored in large amounts in platelets. Recent studies report that platelet-secreted VEGF is a better predictor of tumor progression than serum VEGF.[26] It is conceivable that platelet-secreted VEGF stimulates angiogenesis in vivo, and this is supported by in vitro evidence that platelets stimulate endothelial cell proliferation.[27] Antiplatelet agents such as abciximab can block platelet aggregation and adhesion to fibrin(ogen) and can also inhibit platelet degranulation.[23] By blocking granule release, abciximab inhibits ADP-induced secretion of serotonin, TGF-β, PDGF-AB, and VEGF.[20,23-25] In addition to agonists, tumor cells can induce platelets to secrete VEGF and this secretion is also blocked by abciximab.[20]

Platelets also stimulate sprouting of endothelial cells in in vitro models of angiogenesis, and GpIIb/IIIa antagonists can inhibit platelet-stimulated sprouting.[28] These data suggest that platelet–endothelial cell bridging involves platelet GpIIb/IIIa integrin and platelet degranulation that results in secretion of angiogenic factors such as VEGF and thrombin. Thrombocytosis, an increase in platelet count, is directly correlated with survival of patients with lung and ovarian carcinoma.[29-31] A correlation between thrombocytosis and serum VEGF and IL-6 levels was observed in patients with advanced cancer.[32] Patients with thrombocytosis had a median VEGF serum concentration that was 3.2 times higher ($P < 10^{-4}$) and a median IL-6 serum level that was 5.8 times higher ($P = 0.03$) than in patients without thrombocytosis.[32] Both VEGF and IL-6 have been implicated in growth and angiogenesis, suggesting that platelets could stimulate tumor growth, angiogenesis, and metastasis. In addition to proangiogenic factors, platelets contain antiangiogenic factors such as thrombospondin.[33] For tumor angiogenesis to be sustained there must be a balance between positive and negative regulators of platelet-secreted factors, and detailed understanding of this process should provide insight into tumor angiogenesis.

PLATELETS ACT AS A CLOAKING DEVICE TO PROTECT TUMOR CELLS FROM IMMUNE SURVEILLANCE

The important role of tumor cell–platelet aggregate formation during the hematogenous metastatic cascade is part of the current textbook description of metastases, yet the exact mechanistic basis of this interaction has remained uncertain. Several unrelated studies have concluded that platelets cover the tumor cell to provide a protective coating that can prevent an immune response. Recent studies have begun to dissect the mechanisms involved in tumor cell–platelet interactions. Nieswandt et al[34] reported that natural killer cell–mediated lysis of tumor cells is severely impeded by platelets in vitro and in mouse tumor models. The authors proposed that even when natural killer cells threaten the survival of tumor cells in the blood, platelets are capable of protecting them from cytolysis, thereby promoting metastasis. This concept of surface shielding is supported by another study that elegantly demonstrates that heparin can block tumor cell–platelet interactions in mouse tumor models by inhibiting P-selectin–mediated interactions of platelets with carcinoma cell surface mucins.[35] This study provides a new paradigm for heparin therapy suggesting that heparin should be viewed not just as an anticoagulant but also as an inhibitor of tumor cell–platelet interactions. The authors provide animal data to support that a single dose of heparin can reduce metastasis in a lung colonization mouse model. This suggests that even a short-term exposure with an appropriate antiplatelet agent in the appropriate microenvironment can markedly reduce long-term colonization by tumor cells.

DO PLATELETS CONTRIBUTE TO TUMOR GROWTH AND INVASION?

Because platelet particles contain a variety of proinvasive and promigratory factors, it is conceivable that extravasated platelets that come in contact with the leading edge of the tumor could stimulate cell growth, motility, and invasion. The role of platelets in directly stimulating tumor growth and invasion is not well explored, but there is some evidence suggesting that platelets stimulate breast cancer cell invasion.[36] Tumor cell–platelet bridging via fibrinogen or other proteins may initiate a signaling cascade that could stimulate tumor cell invasion. Integrins GpIIb/IIIa and αvβ3 have been implicated in this process, and antiplatelet mAbs can block tumor cell–platelet interactions in vitro and in vivo (for reviews see references 3 and 37). There is no convincing evidence that platelets directly contribute to tumor growth either in vitro or in vivo. Most in vivo studies of tumor cell–platelet interactions have used the lung colonization models, which may not be entirely representative of metastasis in humans. Preclinical animal studies designed to determine whether inhibition of platelet function can regulate tumor growth and angiogenesis are needed in order to determine whether platelets contribute to tumor growth and invasion.

COULD ANTIPLATELET AGENTS HAVE ANTIANGIOGENIC ACTIVITY?

Results from clinical studies with anticoagulants have been equivocal and were reviewed in detail elsewhere.[2] Several studies with anticoagulants such as heparin, vitamin K antagonists, fibrinolytics, and cyclooxygenase and lipoxygenase inhibitors indicate that no single agent by itself is a potent inhibitor of platelet function. Abciximab, eptifibatide, and tirofiban are potent platelet antagonists, and they can be used to determine whether platelets contribute to tumor growth and metastasis. All three agents block GpIIb/IIIa integrin–mediated platelet aggregation and are approved for patients undergoing percutaneous coronary intervention.[38] Several large cardiovascular clinical studies have demonstrated that these agents are clinically more effective than aspirin or heparin.[38] The reason for the superior antithrombotic activity of platelet GpIIb/IIIa agents compared with aspirin alone is that these agents inhibit platelet aggregation irrespective of the agonist. These agents have been proved to be safe and effective and do not cause excessive bleeding. Use of these agents may help to determine whether blockade of platelet function provides antiangiogenic and antimetastatic benefits.

There are several issues that must be considered regarding chronic administration of antiplatelet agents to patients with cancer. These include a risk of bleeding, thrombocytopenia, and an acquired immune response to platelets and/or to the therapeutic agent. Another issue that needs to be carefully evaluated is whether complete suppression of platelet function is necessary for efficacy and feasible for long-term therapy. Single use of eptifibatide and tirofiban in patients with cardiovascular disease can block platelet function for minutes to hours, and abciximab in a similar patient population can inhibit platelet function for several days.[38] It is reasonable to assume that sustained inhibition of platelet function would be required to inhibit tumor growth and angiogenesis; therefore, repeated administration of these agents will be needed for chronic inhibition of platelet-mediated tumor growth and angiogenesis. In contrast, only a short-term exposure of antiplatelet agents would be required to inhibit hematogenous metastasis.

The currently available platelet GpIIb/IIIa antagonists could be used to determine whether inhibition of platelet function slows tumor angiogenesis and growth. Because platelets contribute to tumor angiogenesis and metastasis and antiplatelet agents do not have a cytotoxic effect, these agents can be tested to determine whether they have antiangiogenic activity. The idea of low-dose continuous therapy for antiangiogenic agents as proposed by Kerbel and others[39] could also be applied to this class of drugs. Recent findings suggest that low-dose continuous administration of antiangiogenic agents such as the antibody to VEGF, when used in combination with vinblastine, is more effective than infrequent monotherapy.[39] It is conceivable that low-dose frequent therapy with antiplatelet agents in combination with cytotoxic therapy may yield an enhanced antitumor effect when compared with monotherapy alone.

Folkman and others[21,40] have postulated that a true antiangiogenic agent should not only inhibit tumor growth but also block hematogenous metastasis, and multiple preclinical studies have demonstrated that inhibition of GpIIb/IIIa blocks experimental metastasis. The murine $F(ab')_2$ version of abciximab, $F(ab')_2$, completely prevented tumor cell–induced thrombocytopenia and lung metastasis in rats.[20] These results in conjunction with many other preclinical studies as discussed earlier suggest that platelets use GpIIb/IIIa to interact with circulating tumor cells and that blockade of this receptor prevents metastasis.

Unlike the compelling evidence that platelets contribute to hematogenous metastasis, there are minimal in vivo published data to discern whether chronic inhibition of platelets can delay tumor growth and angiogenesis. Such studies are clearly needed in order to dissect the exact contribution of platelets to tumor growth and angiogenesis. A few studies suggest that combined blockade of platelet GpIIb/IIIa and the angiogenic $\alpha v\beta 3$ integrin is superior to blockade of just $\alpha v\beta 3$ integrin.[28] Abciximab is distinct from eptifibatide and tirofiban as it binds and blocks both GpIIb/IIIa and $\alpha v\beta 3$ integrin with equivalent affinity. Recent in vivo findings suggest that $mE3F(ab')_2$ has both antiangiogenic and antimetastatic properties.[28] These studies support the idea that antiplatelet therapy is also antiangiogenic. Careful evaluation of antiplatelet therapy in combination with other tumor-debulking therapy such as the use of cytotoxic agents is required to delineate the stage of tumor growth and angiogenesis at which platelets are most involved.

Cancer patients at high risk for developing deep vein thrombosis, disseminated intravascular coagulation, and/or thrombocytosis are probably most likely to benefit from antiplatelet GpIIb/IIIa therapy. An issue currently facing clinical development of antiangiogenic agents such as endostatin, angiostatin, and VEGF antagonists is the lack of approved surrogate markers to monitor efficacy. Monitoring ex vivo platelet aggregation is a well-established technique and can be used as a surrogate marker for monitoring the efficacy of antiplatelet agents in an oncological setting.

CONCLUSION

Tumor cells possess the capacity to interact with all of the compartments of the hemostatic system, activating the coagulation cascade and stimulating the prothrom-

botic properties of platelets and other blood cell components. Platelets are like stealth bombers that carry a large payload of growth factors to the tumor and act as cloaking devices that protect the circulating tumor cells from the immune system and facilitate tumor cell extravasation. These multiple mechanisms contribute to tumor growth, angiogenesis, and metastasis. Therefore, it is possible that blocking tumor cell–platelet interactions can inhibit tumor progression. With the recent development of a new class of potent antiplatelet agents, the GpIIb/IIIa antagonists, we can test whether blocking platelets can inhibit tumor growth. Lessons from earlier clinical studies with the anticoagulants and with antiangiogenic agents that are currently in clinical development suggest that antiplatelet agents should be developed as antiangiogenics and used in combination with other tumor-debulking therapy. Whether such a strategy will be successful merits careful consideration.

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Multiple Roles for Platelet GPIIb/IIIa and αvβ3 Integrins in Tumor Growth, Angiogenesis, and Metastasis

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ABSTRACT

In vivo tumor cells interact with a variety of host cells such as endothelial cells and platelets, and these interactions are mediated by integrins GPIIb/IIIa and αvβ3. We used chimeric (c) 7E3 Fab (ReoPro) and murine (m) 7E3 F(ab′)$_2$ to elucidate the role of these integrins in angiogenesis, tumor growth, and metastasis. These antibodies are potent inhibitors of GPIIb/IIIa and αvβ3. c7E3 Fab inhibited αvβ3-mediated human umbilical vein endothelial (HUVEC) and melanoma cell adhesion, migration, invasion, and basic fibroblast growth factor stimulated proliferation of HUVECs (IC_{50} values range from 0.15 to 5 μg/ml for different assays). In an *in vitro* angiogenesis assay, c7E3 Fab inhibited basic fibroblast growth factor and platelet-stimulated capillary formation of HUVECs (IC_{50} = 10 μg/ml and 15 μg/ml, respectively), demonstrating that endothelial αvβ3 is important for sprouting, and platelet-stimulated sprouting is mediated by GPIIb/IIIa. In an experimental metastasis assay, a single pretreatment of human melanoma cells with c7E3 Fab (2.5 μg/ml) inhibited lung colonization of the tumor cells in severe combined immunodeficient mice. *In vivo*, m7E3 F(ab′)$_2$ partially inhibited growth of human melanoma tumors in nude mice compared with control-treated animals. These data suggest that tumor cell-expressed integrins are important but not the only component involved in tumor growth. Because c7E3 Fab and m7E3 F(ab′)$_2$ do not cross-react with murine integrins, this inhibition of metastasis and tumor growth is attributable to direct blockade of human tumor αvβ3 integrins. m7E3 F(ab′)$_2$ completely blocked tumor formation and growth of human melanoma tumors growing in nude rats. In this xenograft model, m7E3 F(ab′)$_2$ simultaneously binds to both human tumor and host platelet GPIIb/IIIa and endothelial αvβ3 integrins, thus participating as an antiangiogenic and an antitumor agent. Collectively, these results indicate that combined blockade of GPIIb/IIIa and αvβ3 affords significant antiangiogenic and antitumor benefit.

INTRODUCTION

c7E3[2] Fab (abciximab; ReoPro) is a mouse-human chimeric mAb Fab fragment of the parent murine mAb 7E3. c7E3 Fab was the first agent to be approved for use as adjunct therapy for the prevention of cardiac ischemic complications in patients undergoing percutaneous coronary intervention (1). c7E3 Fab binds with high avidity to the GPIIb/IIIa (also known as αIIbβ3) receptor on platelets, which is the major receptor involved in platelet aggregation. c7E3 Fab also binds with equivalent affinity to the vitronectin receptor αvβ3, and it can redistribute between GPIIb/IIIa and αvβ3 receptors *in vitro* (2). We asked whether c7E3 Fab could be used to determine the contribution of platelet GPIIb/IIIa and αvβ3 integrins in tumor growth, angiogenesis, and metastasis.

There is now considerable evidence that progressive tumor growth is dependent on angiogenesis. The formation of new blood vessels provides tumors with nutrients and oxygen, allows the removal of waste products, and acts as conduits for the spread of tumor cells to distant sites (3). Several studies have defined the role of integrins in the angiogenic process (4–6). During the angiogenic process, αvβ3 is up-regulated on the surface of activated endothelial cells, which in turn helps these cells to migrate, proliferate, and invade the tumor (4–6). An antagonist of αvβ3, LM609, suppressed angiogenesis and blocked growth of human tumors that did not express this receptor (7). LM609 was used in a SCID mouse human chimeric angiogenesis model. In this system, αvβ3-negative human melanoma cells were injected into full thickness human skin grafted onto SCID mice. The resulting tumors induced an angiogenic response that enhanced the growth of tumor cells in an orthotopic microenvironment (7). Regular administration of LM609 significantly inhibited growth of αvβ3-negative tumors by blocking the growth of human blood vessels. Because LM609 does not cross-react with mouse integrins, its antiangiogenic activity was attributed to blockade of human αvβ3 receptors in the vasculature of the human skin. A subsequent study using the murine IgG equivalent of c7E3 Fab (m7E3 IgG) in the same model achieved similar results as LM609 (8). Similar to LM609, 7E3 does not cross-react with mouse integrins; therefore, it inhibited growth of human tumors by blocking human αvβ3 receptors in the vasculature of the human skin. In these studies, a partial inhibition of tumor growth was observed, and the combined effect of blocking tumor cell-expressed αvβ3 and endothelial cell-expressed αvβ3 was not evaluated. One limitation of this model is that tumors can grow even in the absence of human vasculature, because the mouse vasculature can sustain tumor growth. To the best of our knowledge, a relevant model examining simultaneous blockade of both host and tumor cell-expressed integrin has not yet been evaluated. One purpose of our study was to evaluate whether combined blockade of host and tumor cell-expressed integrins was superior to blockade of tumor cell-expressed integrins *in vivo*.

The clinical significance of β3 integrin expression in human melanoma was determined in a prospective study that examined the expression of this integrin in patients who were followed for a mean of 98 months after diagnosis with intermediate thickness melanoma (9, 10). This study concluded that tumors in 64% of the patients expressed β3 integrin, and a higher proportion (45%) of patients with β3 positive melanomas were more likely to die of their disease when compared with those with β3 negative tumors (8%).

Angiogenesis can also stimulate the metastatic cascade by providing conduits for the spread of tumor cells to distant sites (6, 11). Some have postulated that platelets are involved in tumor cell extravasation, adherence, or trapping of tumor cell-platelet aggregates to capillary walls, and protection of circulating tumor cells from the antitumor response of the host (reviewed in Refs. 1, 11, 12). The role of platelets in facilitating hematogenous metastasis is well accepted, but little is known about their role in contributing to growth of the primary and/or metastatic tumor. Platelet granules contain a variety of angiogenic factors such as VEGF, platelet-derived growth factor, TGF-β, and fibrinogen, and these modulators are immediately secreted after platelet activation (13). Tumor vasculature is leaky, and extravasated

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[2] The abbreviations used are: c, chimeric; m, murine; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; mAb, monoclonal antibody; HUVEC, human umbilical vein endothelial cell; bFGF, bovine basic fibroblast growth factor; MC, microcarrier; PRP, platelet-rich plasma; PPP, platelet-poor plasma; 3x/wk, three times per week; 5x/week, five times per week; SCID, severe combined immuno-deficient.

fibrin(ogen) that is deposited on the tumor surface can provide an ideal substrate for platelet binding and activation. In addition, tumor cells can activate platelet aggregation (14) and cause the release of VEGF from platelets (15, 16), which in turn can stimulate angiogenesis. c7E3 Fab can block GPIIb/IIIa-mediated platelet aggregation, degranulation, and adhesion to fibrinogen (1). One goal of this study was to determine whether blockade of platelets could inhibit tumor growth *in vivo*. Recently, Verheul *et al.* (17) have demonstrated that platelets stimulate endothelial cell proliferation *in vitro*. Clinically thrombocytosis, an increase in platelet count, is directly correlated with survival of patients of lung and ovarian carcinoma (18–20), supporting the notion that platelets may play a role in tumor growth, angiogenesis, and metastasis. The central hypothesis for our study was that combined blockade of platelet GPIIb/IIIa, endothelial, and tumor cell-expressed αvβ3 could have an enhanced inhibitory effect compared with blockade of tumor cell-expressed αvβ3 alone. c7E3 Fab is one such agent that can antagonize GPIIb/IIIa and αvβ3, and it is widely used in the clinic as an antithrombotic agent. Therefore, we wanted to determine whether c7E3 Fab has anticancer properties. Results from this study indicate that c7E3 Fab and m7E3 $F(ab')_2$, in addition to providing antithrombotic effect, also possess antiangiogenic and antitumor properties.

MATERIALS AND METHODS

Reagents. Bovine bFGF and human $VEGF_{165}$ were obtained from R&D Systems (Minneapolis, MN). mAb 1976Z (LM609), a mAb against integrin αvβ3, and MAB1961 (PIF6), a mAb against integrin αvβ5, were purchased from Chemicon (Temecula, CA). Biocoat cell culture inserts (pore size 8 μm) were purchased from Becton Dickinson (Bedford, MA). Vybrant cell adhesion assay kit (V-13181) was purchased from Molecular Probes (Eugene, OR). Human plasminogen-free fibrinogen (von Willebrand/fibronectin depleted) was purchased from Enzyme Research Labs (South Bend, IN). Bovine skin gelatin was purchased from Sigma (St. Louis, MO). Human vitronectin was purchased from Promega (Madison, WI), and type I collagen from Life Technologies, Inc. (Gaithersburg, MD). c7E3 Fab, m7E3 $F(ab')_2$, and 10E5 were generated at Centocor. For animal experiments m7E3 $F(ab')_2$, instead of the intact IgG, was used to eliminate platelet clearance and any other Fc receptor-mediated events.

Cell Lines. HUVECs were purchased from Clonetics (Walkersville, MA), and cultured in EBM complete medium (Clonetics) containing 10% fetal bovine serum, long R insulin-like growth factor-1, ascorbic acid, hydrocortisone, human epidermal growth factor, human VEGF, gentamicin sulfate, and amphotericin-B. Cells were grown at 37°C and 5% CO_2, and medium was changed every 2–3 days. Cells were passaged when they reached 80% confluence. Passages 3–8 were used in all of the experiments. The A375S2 human melanoma cell line was obtained from American Type Culture Collection (Rockville, MD), and deemed free of *Mycoplasma* and bacterial contaminants. The cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. HT168M1 melanoma cells were isolated from a patient as described (21) and were cultured in 10% FBS and RPMI 1640. Human colon carcinoma HT29 cells were obtained from American Type Culture Collection, and deemed free of *Mycoplasma* and bacterial contaminants. The cells were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, and 0.1 mM nonessential amino acids.

Flow Cytometry. To stain surface integrins, cells were harvested, rinsed, suspended in unsupplemented RPMI 1640, and sequentially incubated for 60 min at room temperature with anti-integrin mAbs (10 μg/ml) and FITC-labeled goat antimouse antibody (1:200). In some instances, cells were directly labeled with FITC-labeled anti-integrin mAbs (10 μg/ml). Absence of primary antibody or substitution of primary antibody with isotype-matched irrelevant antibody served as negative controls. Cells were immediately analyzed with a fluorescence-activated cell sorter Scan II flow cytometer (Becton Dickinson, Mountain View, CA).

Adhesion Assay. Microtiter plates (Linbro-Titertek; ICN Biomedicals, Inc.) were coated at 4°C overnight with vitronectin (1 μg/ml), gelatin (0.1%), fibrinogen (100 μg/ml), type I collagen (10 μg/ml), or fibronectin (10 μg/ml). Fibrin-coated Microtiter wells were formed by thrombin treatment (1 units/ml) of fibrinogen. These concentrations of proteins supported optimal cell adhesion. Immediately before use plates were rinsed with PBS and blocked for 1 h with 1% BSA/PBS (pH 7.4). Adherent cells were labeled with Calcein a.m. fluorescent dye (Molecular Probes) according to the manufacturer's instructions, harvested, washed twice, and suspended in 0.1% BSA in DMEM. After cell density was adjusted to 5×10^5/ml, cells were incubated with various concentrations of antibodies for 15 min at 37°C. The cell-antibody mixture was

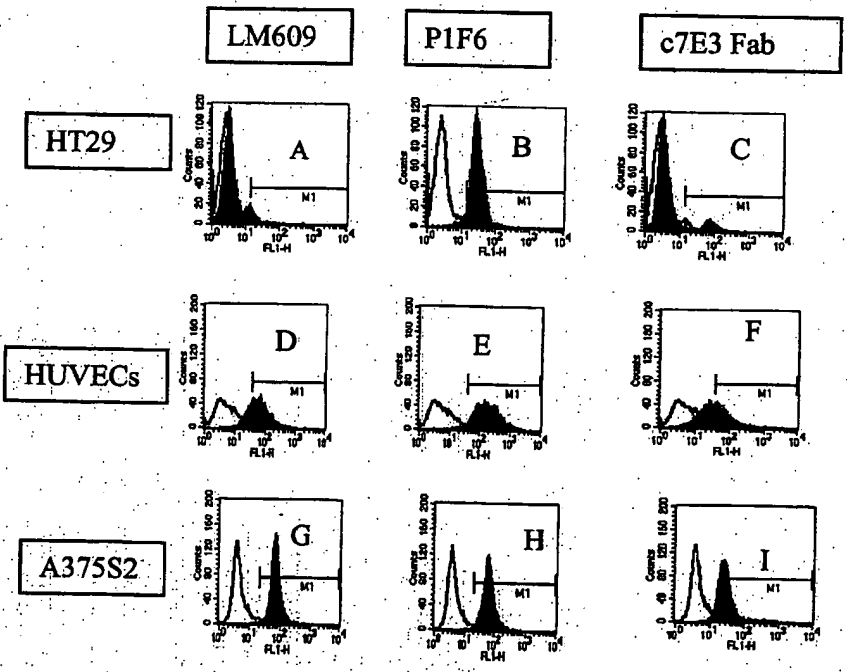

Fig. 1. HT29 cells (*A–C*) express αvβ5 but not αvβ3 integrin on their surface. HUVEC (*D–F*) and A375S2 (*G–I*) cells express αvβ5 and αvβ3 integrin on their surface. Tumor cells and endothelial cells were stained by immunofluorescence and analyzed by flow cytometry. The *histogram on the left* represents background fluorescence in the presence of isotype matched antibody. The *histogram on the right* indicates staining of test antibody. *A, D,* and *G,* LM609 (mAb directed to αvβ3, 10 μg/ml); *B, E,* and *H,* PIF6 (mAb directed to αvβ5, 10 μg/ml); and *C, F,* and *I,* c7E3 Fab (10 μg/ml). *M1,* marker that indicates the gate for positive cells.

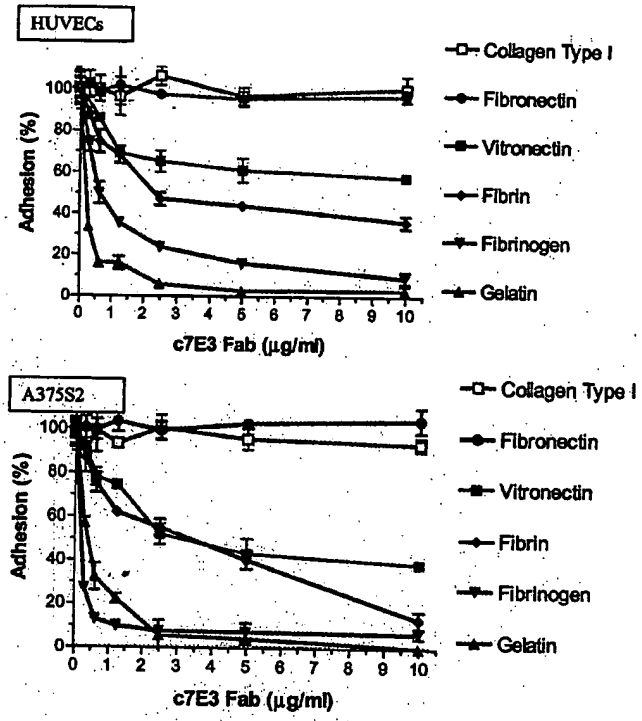

Fig. 2. Adhesion of HUVECs and A375S2 melanoma cells to matrix proteins. Adhesion assay was performed as described in "Materials and Methods." Cell adhesion to BSA-coated wells served as a negative control. Extent of cell adhesion in the presence of various concentrations of antibody was plotted as a percentage of cell adhesion in the absence of antibody that was considered as 100%. Each data point is the mean of triplicate determinations and is representative of at least three experiments; *bars*, ± SD.

added to wells (100 μl/well) and incubated for 1 h at 37°C. Plates were rinsed twice with PBS to remove unbound cells, and adhesion was measured in a fluorescence plate reader (Fluoroskan; Tecan, Research Triangle Park, NC) at 485–538 nm. Cell adhesion to BSA-coated wells served as a negative control. Isotype-matched antibodies served as a negative control.

Cell Migration Assay. Cell migration assays were performed in 24-transwell chambers with a polystyrene membrane (6.5-mm diameter, 10-μm thickness, and 8-μm pore size) as described previously (22). Briefly, the underside of the membrane was coated with vitronectin (2 μg/ml) for 60 min at room temperature and then blocked with a solution of 1% BSA/PBS at room temperature for 60 min. Next, membranes were washed with PBS and dried. Serum-free medium (750 μl) containing 0.1% BSA and bFGF (20 ng/ml) or medium containing 10% FBS was added to the lower chambers. Subconfluent 24-h cultures were harvested with trypsin-EDTA, washed twice, and resuspended in serum-free medium. Cells (100,000/500 μl) were added to the upper chambers in the presence or absence of antibodies. The chambers were placed in a tissue culture incubator, and migration was allowed to proceed for 4–6 h. Migration was terminated by removing the cells on the top with a cotton swab, and the filters were fixed with 3% paraformaldehyde and stained with Crystal Violet. The extent of cell migration was determined by light microscopy, and images were analyzed using the Phase 3 image analysis software (Glen Mills, PA). The software analyzes the total area occupied by the stained cells on the bottom side of the filter, and this is directly proportional to the extent of cell migration.

Invasion Assay. The cell invasion assays were performed as described (23). Briefly, fibrinogen (plasminogen-free 100 μl of 10 mg/ml) and 100 μl of 1 unit/ml thrombin was mixed, and immediately added to the top chamber of 24-well transwell plates (6.5-mm diameter, 10-μm thickness, and 8-μm pore size). The plates were incubated at 37°C for 30 min to form a fibrin gel. Confluent tumor cells (A375S2) were trypsinized, centrifuged, resuspended in basal medium supplemented with 0.1% BSA and 10 μg/ml plasminogen (Enzyme Research Labs) with various concentrations of antibodies, and incubated for 15 min at room temperature. Cells (100,000/500 μl) were added to the upper chamber in the presence or absence of antibodies. The lower compartment of the invasion chamber was filled with 0.75 ml of 10% FBS-DMEM, which served as a chemoattractant, and the plate was transferred to a tissue culture incubator. After 24 h, invasion was terminated by removing the cells on the top with a cotton swab, and the filters were fixed with 3% paraformaldehyde and stained with Crystal Violet. The extent of cell migration was analyzed using the Phase 3 image analysis software as described above.

Endothelial MC-based Sprouting Assay. A modification of the methods of Nehls and Drenckhahn (24) was used to measure capillary tube formation in three-dimensional fibrin-based matrix. Gelatin-coated cytodex-3 MCs (Sigma) were prepared according to recommendations of the supplier. Freshly autoclaved MCs were suspended in EBM-2 + 20% FBS, and endothelial cells were added to a final concentration of 40 cells/MC. The cells were allowed to attach to the MCs during a 4-h incubation at 37°C. The MCs were then suspended in a large volume of medium and cultured for 2–4 days at 37°C in 5% CO_2 atmosphere. MCs were occasionally agitated to prevent aggregation of cell coated beads. MCs were embedded in a fibrin gel that was prepared as follows: human fibrinogen (2 mg/ml) was dissolved in plain medium containing antibodies and/or bFGF, PRP containing 250,000 platelets/μl, PPP, or serum containing EBM-2 medium. PRP, PPP, and gel-filtered platelets were prepared from citrated whole blood obtained from healthy volunteers as described (2). To prevent excess fibrinolysis by fibrin-embedded cells, aprotinin was added to the fibrinogen solution and to growth medium at 200 units/ml. Cell-coated MCs were added to the fibrinogen solution at a density of 100–200 MCs/ml (50–100 beads/per 48-well plate), and clotting was induced by addition of thrombin (0.5 units/ml). After clotting was complete, 0.5 ml of solution (containing all of the components described above except fibrinogen and thrombin) was added to the fibrin matrices. The plates were incubated at 37°C

Table 1 *Adhesion of HUVECs and A375S2 to vitronectin, gelatin, fibrinogen, fibrin, fibronectin, and type I collagen*

Extent of cell adhesion in the presence of various concentration of antibody was plotted as a percentage of cell adhesion in the absence of antibody that was considered as 100%. Each data point is the mean of triplicate determinations (±SD). The concentration of antibodies used was 10 μg/ml.

A. Adhesion of HUVECs (%) ±SD

	Vitronectin	Gelatin	Fibrinogen	Fibrin	Fibronectin	Type I collagen
Human IgG	96.3 ± 11.4	109.0 ± 8.8	108.0 ± 6.3	99.7 ± 4.5	96.8 ± 4.7	99.3 ± 4.1
LM609	26.3 ± 3.7	36.5 ± 4.7	14.3 ± 2.5	48.1 ± 1.5	102.8 ± 7.2	108.8 ± 12.7
PIF6	39.8 ± 5.9	94.4 ± 15.1	94.5 ± 4.2	96.7 ± 4.5	103.2 ± 3.8	115.7 ± 8.1
LM609 + PIF6	3.7 ± 0.4	32.2 ± 5.2	10.7 ± 1.1	30.7 ± 8.9	99.6 ± 4.7	116.2 ± 4.1
c7E3 Fab	54.9 ± 0.9	2.5 ± 2.3	8.7 ± 2.9	35.8 ± 3.0	96.3 ± 2.8	99.6 ± 6.0

B. Adhesion of A375S2 (%) ±SD

	Vitronectin	Gelatin	Fibrinogen	Fibrin	Fibronectin	Type I collagen
Human IgG	104.0 ± 5.3	94.6 ± 12.4	102.5 ± 5.9	99.5 ± 4.0	100.0 ± 5.5	99.1 ± 3.3
LM609	42.1 ± 6.1	25.2 ± 7.1	14.0 ± 1.8	50.0 ± 1.9	104.0 ± 8.1	100.0 ± 1.5
PIF6	28.5 ± 3.8	87.4 ± 7.8	99.4 ± 3.6	92.9 ± 4.7	101.0 ± 5.7	101.0 ± 7.3
LM609 + PIF6	0.9 ± 0.3	1.1 ± 1.5	10.3 ± 2.6	47.6 ± 3.2	109.0 ± 4.1	102.0 ± 4.6
c7E3 Fab	38.1 ± 0.7	6.0 ± 1.0	6.5 ± 2.1	12.9 ± 3.8	104.0 ± 5.6	93.1 ± 3.1

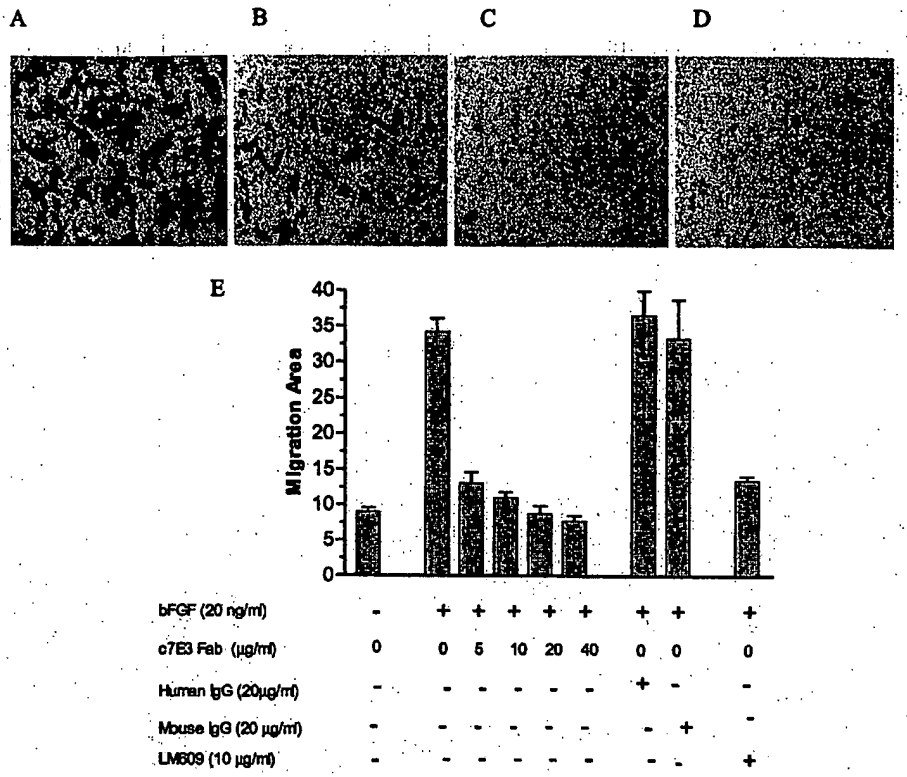

Fig. 3. Migration of HUVECs toward vitronectin in the presence of bFGF. The undersides of migration chamber filters were coated with 2 μg/ml of vitronectin, and the assay was performed as described in "Materials and Methods." Cells were allowed to migrate for 6 h. Each data point is the mean of 3 transwell filters; *bars*, ± SD. Digital photomicrographs of endothelial cell migration in the presence of *A*, bFGF (20 ng/ml) + control antibody (20 μg/ml); *B*, bFGF (20 ng/ml) + c7E3 Fab (5 μg/ml); *C*, bFGF (20 ng/ml) + c7E3 Fab (40 μg/ml); and *D*, control antibody (20 μg/ml) and absence of bFGF. *E*, graphical representation of inhibition of cell migration in the presence of various antibodies.

and 5% CO_2 for 1–3 days. After 1–3 days, gels were fixed with a solution of 3% paraformaldehyde in PBS, and the number of capillary sprouts with length exceeding the diameter of the MC bead (150 μm) was quantified by using the Phase 3 image analysis.

Endothelial Cell Proliferation and Apoptosis Assays. Subconfluent HUVECs were trypsinized, washed, and resuspended in complete medium. Cells (5000) were added to each well of 96-well plates. To test whether the plates themselves may influence the assay, endothelial cells were plated on normal tissue culture plates, high protein-binding plates that were precoated with vitronectin (1 μg/ml), gelatin (0.1%), or type I collagen (2 μg/ml). Cells were allowed to attach for 2 h, medium was aspirated, wells were washed once with PBS, and 100 μl of medium (0.1% serum-M199 or 2% serum-M199) containing bovine bFGF-2 (R&D systems), human recombinant $VEGF_{165}$ ($r + D$ Systems), and/or various antibodies was added to each well. The plates were incubated at 37°C for 48 h. Extent of cell proliferation was determined by the Celltiter 96 Aqueous kit (Promega), ATP kit (Packard, Meridian, CT), or BrdUrd kit (Oncogene Research Products). For the MTS and the BrdUrd assay, absorbance was measured at 490 nm and 540/450 nm, respectively. Luminescence intensity was measured for the ATP assay in a TopCount reader (Packard). To quantify apoptosis, cells were treated as above with antibodies or positive control etoposide for 18 h, and extent of DNA fragments were measured by using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany).

Matrigel-based Angiogenesis Assay in Nude Rats. The Matrigel plug-based angiogenesis assay was performed as described earlier (25) with slight modifications. Briefly, cold Matrigel (Becton Dickinson) was mixed with bFGF (5 μg/ml) and m7E3 $F(ab')_2$ (~300 μg/ml) or an equal volume of PBS. The next day, 2 ml of Matrigel solution was injected s.c. into nude rats (Taconic, Germantown, NY), and animals were dosed i.p. with 6 mg/kg of m7E3 $F(ab')_2$. This dose of m7E $F(ab')_2$ completely inhibits rat platelet aggregation *ex vivo* (26). Animals were dosed every day for 6 days, and plugs were removed on day 7. The extent of angiogenesis was quantified by using the Drabkins kit (Sigma) as described (25).

Lung Metastasis Assay. The lung metastasis assay was performed as described previously (27). Human melanoma HT168M1 cells were pretreated with 2.5 μg/ml of c7E3 Fab or control antibody for 15 min at room temperature, washed, and 1×10^6 cells were tail vein injected into female SCID mice. After 1 month, animals were euthanized, lungs were removed and fixed in paraformaldehyde, and the number of lung colonies were counted.

Growth of Human Melanoma Tumors in Nude Mice and Nude Rats. To determine whether m7E3 $F(ab')_2$ could inhibit tumor growth *in vivo*, we used a human melanoma xenograft model in nude mice and nude rats. Briefly, A375S2 cells (3×10^6/animal) were s.c. injected into female nude mice (Charles River, Raleigh, NC) or nude rats (Taconic). Tumor cells were pretreated with antibody (100 μg/ml for 5 min) before injection or therapy was initiated after animals had developed measurable tumors. Antibody was injected i.p. at a dose of 200 μg/animal or at an animal body weight-adjusted dose of 3–10 mg/kg. Control groups were injected with equivalent volume of diluent (PBS). Tumor volume (mm^3) was calculated based on the formula: (length × width × width)/2 and tumor wet weight (mg) was obtained at termination of the study.

RESULTS

Endothelial and Tumor Cell Adhesion to Matrix Proteins. Flow cytometry was used to characterize integrin expression. A375S2 and HUVECs expressed both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, whereas HT29 cells expressed $\alpha v\beta 5$ but not $\alpha v\beta 3$ (Fig. 1). A375S2 and HUVECs but not HT29 cells stained with c7E3 Fab (Fig. 1). Therefore, we used A375S2 and HUVECs to determine the effects of $\alpha v\beta 3$ blockade in tumor growth and angiogenesis.

Because $\alpha v\beta 3$ binds gelatin, fibrinogen, fibrin, and vitronectin (28, 29), we questioned whether c7E3 Fab could block cell adhesion to these matrix proteins. c7E3 Fab completely inhibited adhesion of HUVECs and A375S2 cells to fibrinogen and gelatin, and it partially inhibited cell adhesion to vitronectin (Fig. 2*A*; Table 1). c7E3 Fab completely inhibited tumor cell adhesion to fibrin, whereas it partially blocked endothelial cell adhesion to fibrin (Fig. 2; Table 1), suggesting that endothelial cells use more than the $\alpha v\beta 3$ receptor to adhere to fibrin. Because HT-29 cells do not express $\alpha v\beta 3$-integrin, c7E3

Fab did not block cell adhesion (data not shown). Collectively, the data indicate that αvβ3 mediates cell adhesion (Figs. 2 and 3; Table 1).

Migration of Human Melanoma and Endothelial Cells. Results described above indicate that c7E3 Fab blocked αvβ3-mediated cell adhesion, therefore we determined whether c7E3 Fab could block cell migration. c7E3 Fab dose dependently inhibited bFGF-stimulated endothelial cell migration (Figure 3). Interestingly, c7E3 Fab also inhibited migration of A375S2 when serum was used as a chemoattractant (Figure 4). Although c7E3 Fab only partially inhibited cell adhesion to vitronectin (Figure 2), it completely blocked cell migration towards this matrix protein. Similar results were obtained with LM609, P1F6 and the combination of both antibodies (Table 1). 10E5 did not block migration of A375S2 cells, suggesting that GPIIb/IIIa is not functionally expressed in this tumor cell line. These findings suggest that endothelial and melanoma cells primarily use αvβ3 integrin to migrate towards vitronectin, and c7E3 Fab can inhibit both bFGF and serum stimulated cell migration.

Human Melanoma Cell Invasion through Fibrin. Because c7E3 Fab inhibited cell adhesion and migration, we determined whether this antibody could block invasion of tumor cells. Invasion is a multistep process that involves cell adhesion, degradation of the matrix, and migration of cells through the degraded matrix. We chose fibrin as a matrix for tumor cells because peritumoral deposition of fibrin *in vivo* facilitates tumor cell extravasation and hematogeneous spread (30). Invasion of A375S2 cells was inhibited by LM609 (Fig. 5), suggesting the involvement of at least αvβ3 in this process. Similarly, c7E3 Fab dose-dependently inhibited tumor cell invasion through fibrin. P1F6 was only partially effective at inhibiting tumor cell invasion, and no enhanced inhibition was observed when it was combined with LM609, suggesting that αvβ5 is involved to a lesser degree than αvβ3 in tumor cell invasion. Irrelevant IgG and a mAb directed to platelet GPIIb/IIIa (10E5) served as negative controls. Collectively, these data suggest that blockade of αvβ3 by c7E3 Fab can effectively block invasion of human melanoma cells.

c7E3 Fab Inhibits Proliferation of Endothelial Cells by Promoting Apoptosis. Because angiogenesis involves not only endothelial cell adhesion, migration, and invasion, but also endothelial cell proliferation, we asked whether c7E3 Fab could inhibit proliferation of endothelial cells. c7E3 Fab dose-dependently blocked endothelial cell proliferation stimulated by bFGF and serum (Fig. 6). c7E3 Fab inhibited cell proliferation by inducing apoptosis of proliferating endothelial cells (Fig. 6*B*). No effect of the drug was observed on quiescent endothelial cells, suggesting that αvβ3 function is only essential for proliferating endothelial cells. These findings indicate that c7E3 Fab inhibits endothelial cell proliferation in response to serum and bFGF, suggesting that αvβ3 plays a central role in mediating endothelial cell proliferation.

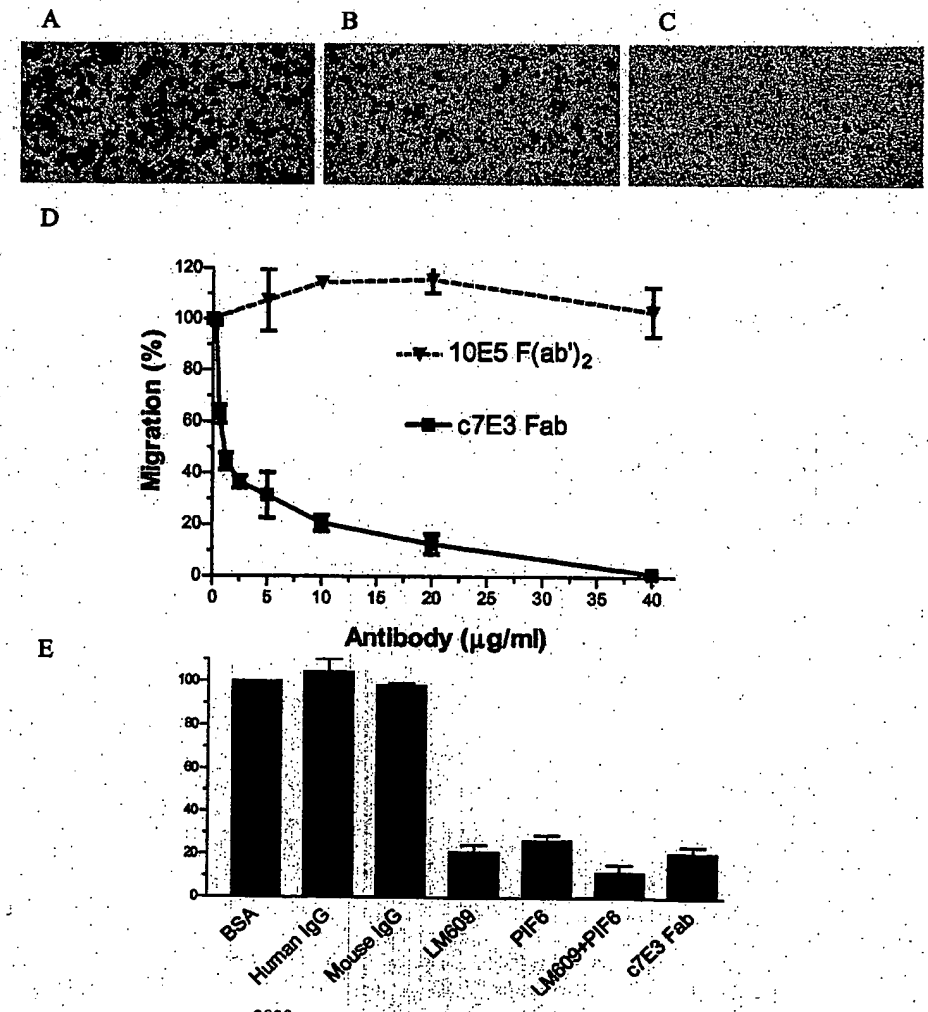

Fig. 4. Migration of A375S2 cells toward 10% FBS. Migration assay was allowed to proceed for 4 h, and the data were captured as described in "Materials and Methods." Digital photomicrographs of tumor cell migration in: (*A*) absence, or presence of c7E3 Fab (*B*) 5 μg/ml and (*C*) 20 μg/ml. *D*, graphical representation of cell migration in the presence of various concentrations of c7E3 Fab or 10E5 F(ab')$_2$. *E*, graphical representation of cell migration in the presence of 10 μg/ml of various antibodies or BSA. The data were normalized to percentage of control (BSA), which was considered as 100%, and each point is the mean of three transwell filters; *bars*, ± SD.

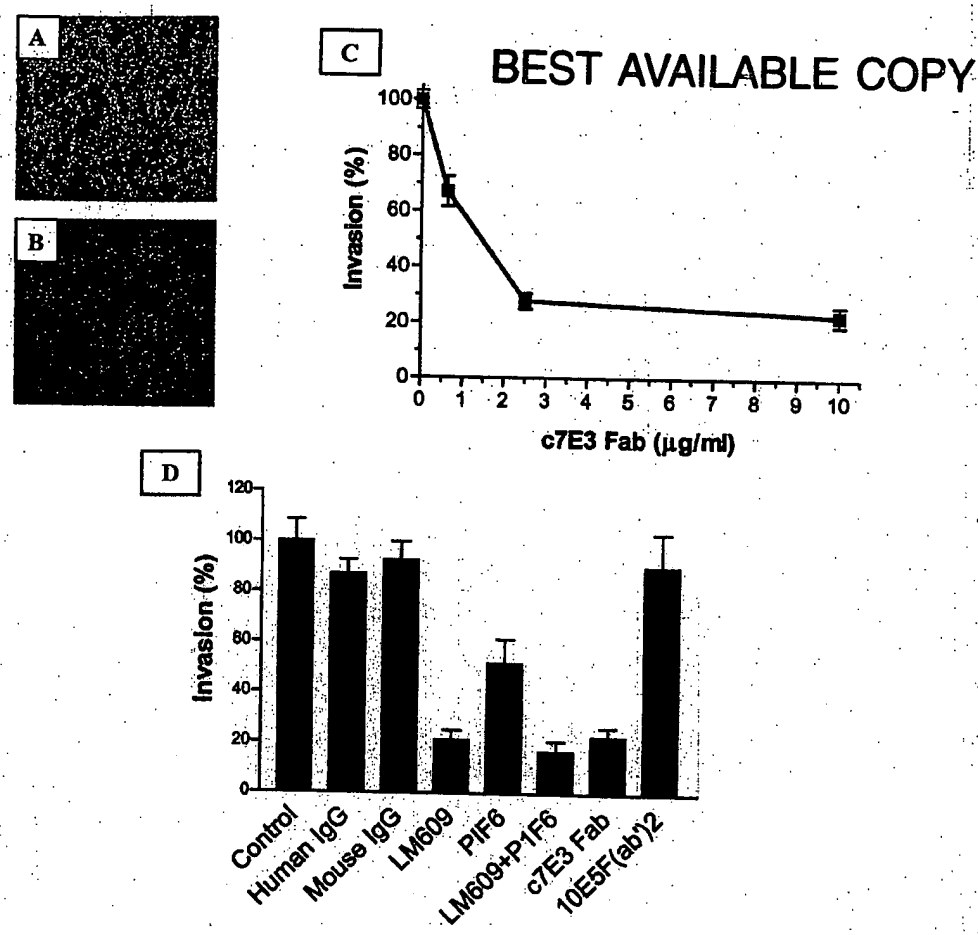

Fig. 5. Invasion of A375S2 cells through a fibrin gel. Invasion was allowed to proceed for 24 h, and the assay was performed as described in "Materials and Methods." Photomicrographs are representative fields (×4 objective lens) of cell invasion in (*A*) the absence of antibodies or (*B*) c7E3 Fab (10 μg/ml). *C*, dose-dependent inhibition of tumor cell invasion by c7E3 Fab. *D*, invasion in presence of various antibodies at (10 μg/ml). The data were normalized to percentage of control (no antibody), which was considered as 100%, and each point is the mean of three transwell filters and the graphs are representative of at least three separate experiments; *bars*, ± SD.

Inhibition of Endothelial Cell Sprouting. Endothelial cell sprouting in a three-dimensional fibrin gel is highly representative of angiogenesis. This assay involves endothelial cell proliferation, adhesion, spreading, migration, and invasion of endothelial cells. To confirm and extend the findings described above, we determined whether c7E3 Fab could block experimental angiogenesis in this assay. c7E3 Fab dose-dependently blocked bFGF stimulated endothelial sprouting in a fibrin gel (Fig. 7*B*). Isotype-matched irrelevant mouse and human antibodies served as negative controls.

In addition to bFGF, several other factors such as VEGF and TGF-β can stimulate endothelial cell proliferation. Platelet granules contain many growth factors including VEGF (13) that are secreted on platelet activation and aggregation. c7E3 Fab blocks VEGF release from platelets *in vitro* (15, 16). Therefore, we explored whether platelets could stimulate endothelial cell sprouting and whether c7E3 Fab could block this effect. Fig. 7*C* indicates that PRP stimulated endothelial sprouting to a greater extent than PPP, suggesting that platelets can stimulate angiogenesis. c7E3 Fab completely inhibited PRP-stimulated endothelial cell sprouting; however, because c7E3 Fab also blocks αvβ3, it was difficult to interpret whether platelet GPIIb/IIIa was involved in platelet-stimulated endothelial sprouting. The involvement of GPIIb/IIIa was demonstrated by the observation that 10E5, a mAb to GPIIb/IIIa, completely blocked gel-filtered platelet-stimulated endothelial cell sprouting (Fig. 7*D*). Inhibition of both endothelial αvβ3 and platelet GPIIb/IIIa receptor inhibited endothelial sprouting that was stimulated by either platelets or by angiogenic factors contained in plasma such as bFGF.

c7E3 Fab Inhibits Experimental Metastasis of Human Melanoma Tumors. Earlier studies indicated that m7E3 F(ab')$_2$ recognizes rat integrins but not murine integrins, and it blocks experimental metastasis of mouse tumor cells in a rat (15). The proposed antimetastatic mechanism that explains these results is that the antibody blocks the host (rat) platelet GPIIb/IIIa and αvβ3 integrins, thereby preventing seeding of the murine tumor cells in the lung endothelium. To test if blockade of tumor cell expressed αvβ3 without inhibiting host integrins could inhibit lung metastasis, we chose a lung colonization model of human melanoma metastasis in SCID mice. In this model, c7E3 Fab only binds to the human tumor cell expressed integrin but not to the host (mouse) integrin. A single pretreatment of human melanoma HT168M1 cells with c7E3 Fab (2.5 μg/ml) significantly decreased the number and size of tumor colonies in the mouse lung (Fig. 8). These results collectively suggest that blockade of tumor cell αvβ3 can provide antimetastatic benefit by blocking tumor cell-platelet, tumor cell-endothelium, and platelet-endothelium interactions.

Inhibition of bFGF-stimulated Angiogenesis in Nude Rats. Results described above indicate that c7E3 Fab is a potent antimetastatic agent *in vivo* and an antiangiogenic agent *in vitro*. Next, we determined whether endothelial αvβ3 and platelet GPIIb/IIIa integrins were involved in angiogenesis *in vivo*. c7E3 Fab does not cross-react with mouse or rat integrins; however, m7E3 F(ab')$_2$ cross-reacts with rat integrins (26); therefore, we evaluated its antiangiogenic activity *in vivo* using a bFGF-stimulated Matrigel plug angiogenesis model. m7E3 F(ab')$_2$ at 6 mg/kg completely inhibited *ex vivo* rat platelet

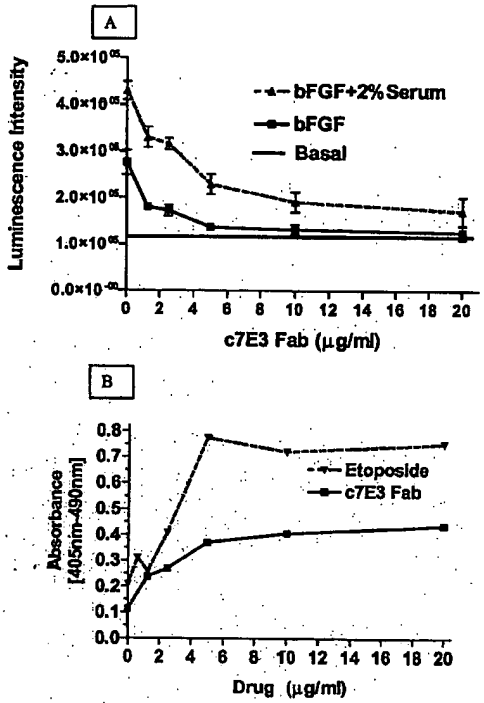

Fig. 6. c7E3 Fab inhibits cell proliferation by promoting apoptosis. Cell proliferation and apoptosis assays were performed as described in "Materials and Methods." *A*, c7E3 Fab inhibits bFGF and bFGF +2% serum stimulated proliferation of human endothelial cells. The data are plotted as luminescence intensity, which is directly proportional to cell number. Basal represents luminescence intensity observed in the absence of bFGF and serum. *B*, c7E3 Fab promotes apoptosis of proliferating endothelial cells. HUVECs were cultured in the presence of bFGF as described in *A* and various concentrations of the positive control (etoposide) or c7E3 Fab. Absorbance on the *Y* axis is directly proportional to extent of apoptosis. Each point represents the mean of triplicate determinations; *bars*, ± SD.

aggregation (26); therefore, this dose of the antibody was used to determine whether m7E3 $F(ab')_2$ could inhibit bFGF-stimulated angiogenesis. The antibody was administered daily for 1 week, Matrigel plugs were removed, and hemoglobin content indicated that m7E3 $F(ab')_2$ significantly inhibited angiogenesis in nude rats (Fig. 9). These data demonstrate that blockade of rat $\alpha v\beta 3$ and GPIIb/IIIa can inhibit angiogenesis in rats.

m7E3 $F(ab')_2$ Inhibits Growth of Human Melanoma Tumors in Nude Mice and in Nude Rats. Because c7E3 Fab inhibits human melanoma cell adhesion and invasion *in vitro*, we explored whether m7E3 $F(ab')_2$ could inhibit tumor growth independent of blocking angiogenesis in a human melanoma xenograft model in nude mice. In this model, m7E3 $F(ab')_2$ only blocks human tumor integrins but not mouse integrins. Antibody therapy (10 mg/kg) was initiated 3 days after tumor cell inoculation, and the dosing regimen was three times per week for the duration of the study. Fig. 10 indicates that m7E3 $F(ab')_2$ partially inhibited growth of human melanoma tumors in nude mice ($P = 0.0002$). These results provide direct evidence that blockade of human melanoma cell-expressed $\alpha v\beta 3$ integrin, without inhibiting mouse $\beta 3$ integrins, can partially inhibit tumor growth *in vivo*.

We hypothesized that combined blockade of both tumor cell-expressed $\alpha v\beta 3$ and the host $\beta 3$ integrins (platelet GPIIb/IIIa and endothelial $\alpha v\beta 3$) may result in enhanced inhibition of tumor growth *in vivo*. To test this hypothesis, the same human melanoma A375S2 cells used in the mouse studies were used in a rat model where m7E3 $F(ab')_2$ blocks multiple integrins: platelet GPIIb/IIIa, endothelial $\alpha v\beta 3$, and tumor cell $\alpha v\beta 3$. This model mimics the clinical situation where the relevant integrins are expressed by the tumor and the host. A dose of 6 mg/kg of m7E3 $F(ab')_2$ was used, because at this concentration the antibody completely inhibited *ex vivo* rat platelet aggregation (26) and inhibited angiogenesis in nude rats (Fig. 9). Two series of experiments were performed in nude rats. In the first experiment, antibody and tumor cells were coinoculated into nude rats, and

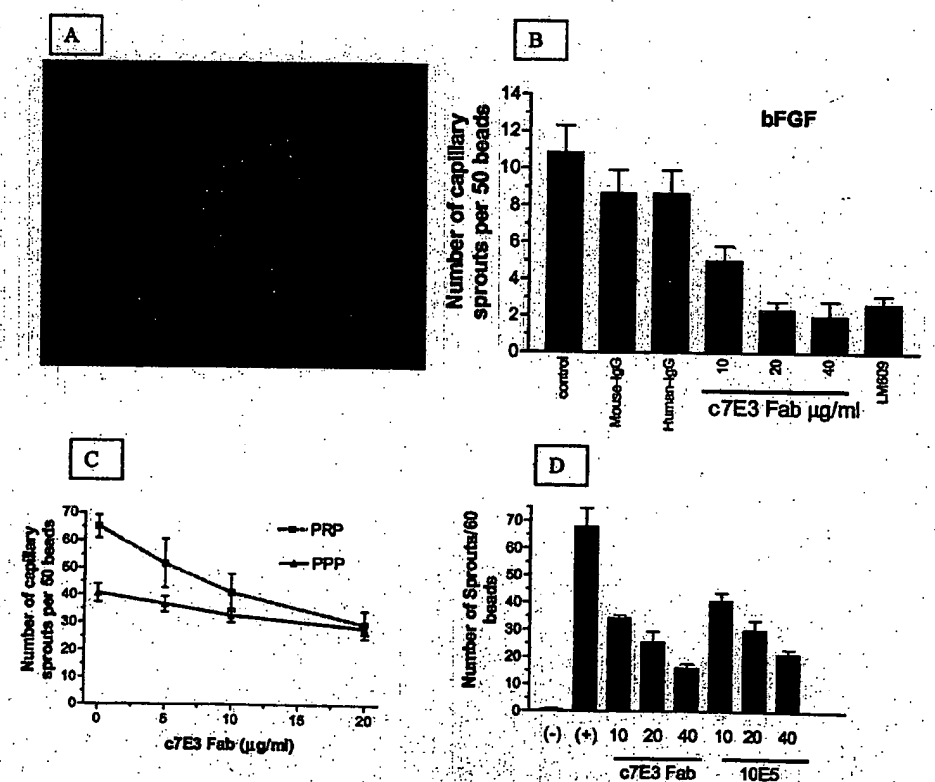

Fig. 7. c7E3 Fab inhibits sprouting of human endothelial cell. *A*, digital photomicrograph of a representative bead coated with HUVECs cultured in a fibrin gel. The number of microvessels sprouting from the bead (total sprouts/50 beads) was quantified as described in "Materials and Methods." *B*, effect of c7E3 Fab on bFGF-stimulated endothelial sprouting. *Control bar* represents sprouting in the absence of antibody. Mouse IgG and human IgG were used at 20 μg/ml as negative control antibodies, and LM609 was used at 20 μg/ml. *C*, dose-dependent inhibition of endothelial sprouting by c7E3 Fab in the presence of PRP and PPP. *D*, dose-dependent inhibition of endothelial cell sprouting by c7E3 Fab and 10E5 in the presence of gel-filtered platelets. −, sprouting in the absence of gel-filtered platelets; +, maximum sprouting in the presence of platelets and mouse IgG (20 μg/ml). Each data point is mean of at least triplicate determinations, and the graphs are representative of two separate experiments; *bars*, ± SD.

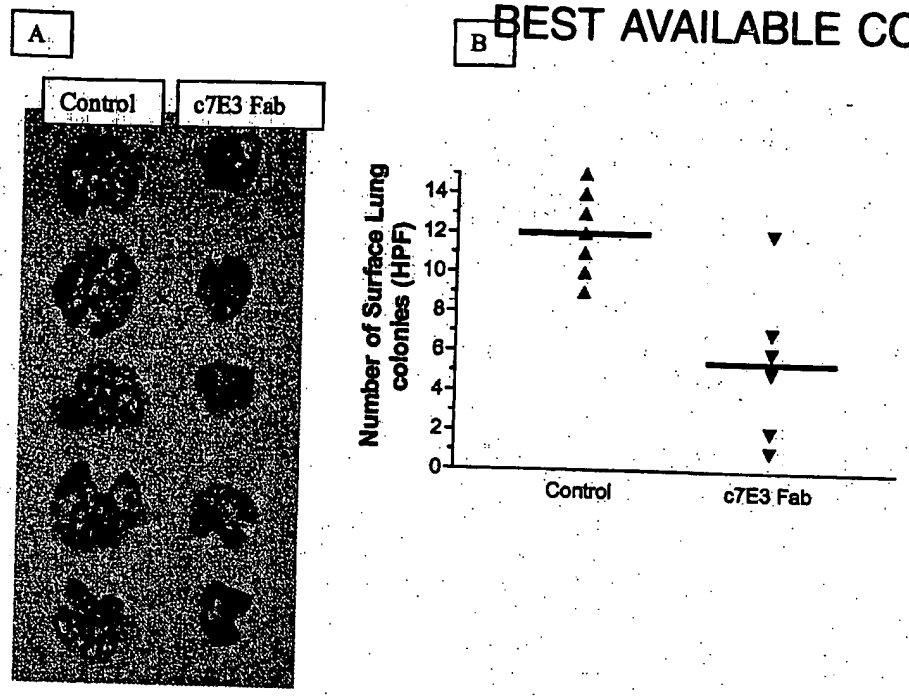

Fig. 8. Effect of c7E3 Fab on lung metastasis of human melanoma cells. Human melanoma HT168M1 cells were incubated with control antibody (2.5 μg/ml) or c7E3 Fab (2.5 μg/ml) for 30 min at room temperature, cells were centrifuged, resuspended, and 1×10^6 cells were tail vein injected into male SCID mice. After 2 months, animals were euthanized, and lungs were removed as described in "Materials and Methods." *A*, photographs of representative lungs treated with control antibody or c7E3 Fab. *B*, the number of surface lung colonies were counted under stereomicroscope. Each data point represents one animal, and the line is the median of the data points. A one-tailed *t* test analysis indicated that c7E3 Fab significantly decreased the number of tumor colonies on the lung surface ($P = 0.0015$) and the weight of tumor-bearing lungs ($P = 0.0463$, data not shown).

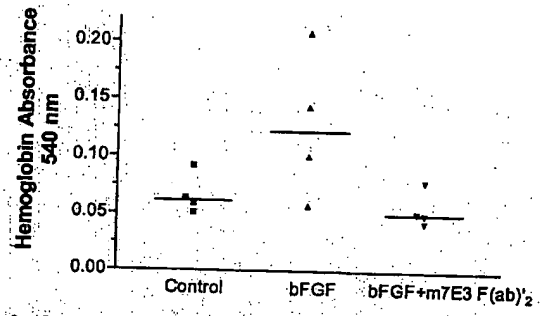

Fig. 9. m7E3 F(ab')$_2$ inhibits bFGF-stimulated angiogenesis in Matrigel plugs in nude rats. The Matrigel plug angiogenesis assay was performed as described in "Materials and Methods." Each point represents hemoglobin content from a Matrigel plug obtained from one animal, and the line represents the median of all values within the group. A one-tailed *t* test analysis indicated that m7E3 F(ab')$_2$ significantly decreased bFGF-mediated angiogenesis ($P = 0.034$).

antibody therapy was administered either 3x/wk or 5x/wk for the duration of the study. Tumor formation and growth were dramatically inhibited in both antibody treated groups (Fig. 11). Animals treated with m7E3 F(ab')$_2$ 3x/wk developed measurable tumors 10 days later compared with the control group, and animals treated with the antibody 5x/wk did not develop tumors throughout the course of the study. Only 50% of the animals in the 3x/wk antibody treatment group developed tumors, and these tumors were significantly smaller compared with the control group ($P < 0.001$; Fig. 11).

To determine whether m7E3 F(ab')$_2$ could inhibit growth of preformed tumors in nude rats, human melanoma A375S2 cells were inoculated into nude rats, tumors were allowed to grow up to ~150 mm^3, and animals were randomized and then treated with m7E3 F(ab')$_2$ (3 mg/kg, daily i.p. administration for the duration of the study) or vehicle control. Assays performed on terminal blood samples demonstrated that m7E3 F(ab')$_2$ inhibited *ex vivo* platelet aggregation and did not cause thrombocytopenia in any of the animals (data not shown; Ref. 26). m7E3 F(ab')$_2$ was administered more frequently in rats compared with the mice, because it has a much shorter circulating half-life in rats. Approximately 150 μg/ml of m7E3 F(ab')$_2$ was detected in the mouse serum the day after the last dose, whereas ~3 μg/ml of circulating antibody was measured in the rat serum the day after the last dose (data not shown), suggesting that the antibody has a shorter circulating half-life in rats compared with mice. Yet, m7E3 F(ab')$_2$ completely prevented growth of preformed tumors in the rat model (Fig. 11) but only had a partial effect in the mouse model (Fig. 10). Collectively, these data provide evidence that combined antitumor and antiangiogenic targeting by m7E3 F(ab')$_2$ is superior than antitumor targeting alone.

DISCUSSION

The salient findings of this study are that platelet GPIIb/IIIa, and endothelial and tumor cell-expressed $\alpha v\beta 3$ participate in angiogenesis, tumor growth, and metastasis. Combined blockade of these receptors on three cell types was more effective at inhibiting tumor

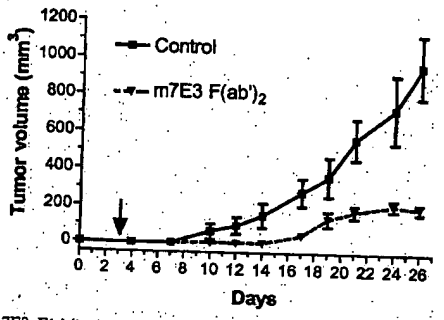

Fig. 10. m7E3 F(ab')$_2$ inhibits growth of human melanoma tumors in nude mice. Human melanoma A375S2 cells (2×10^6) were injected s.c. into female nude mice, and m7E3 F(ab')$_2$ (10 mg/kg 3x/week) or vehicle control therapy was initiated 3 days later as indicated by the *arrow*. Tumor volume measurements were recorded as described in "Materials and Methods." Data points are mean tumor volumes from 10 animals/group; *bars*, ± SD. A one-tailed *t* test analysis indicated that m7E3 F(ab')$_2$ treatment partially inhibited tumor growth on day 26 when compared with control-treated tumors ($P = 0.0002$).

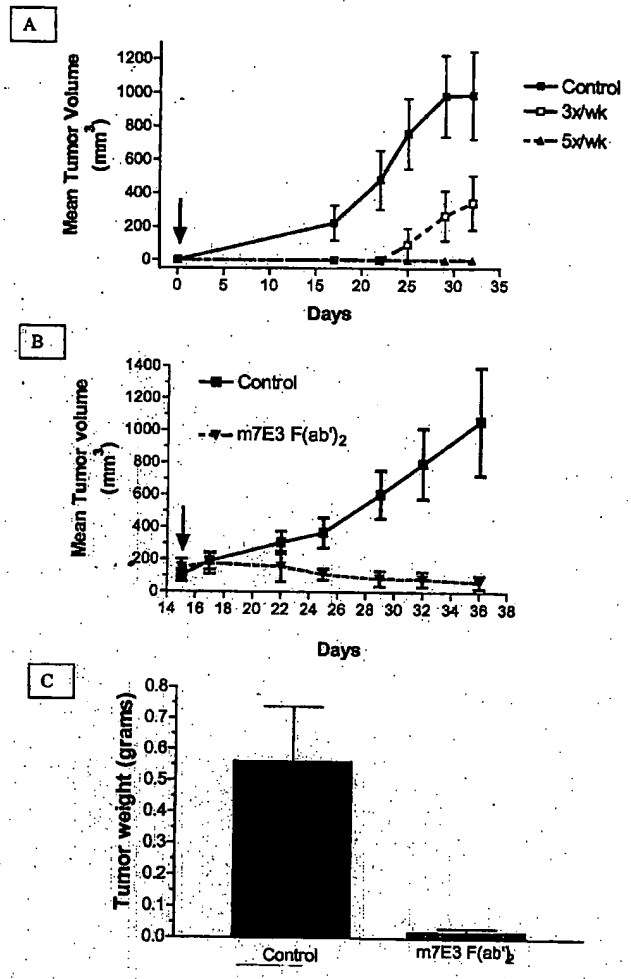

Fig. 11. m7E3 F(ab')$_2$ inhibits growth of human melanoma tumors in nude rats. Human melanoma cells were treated as described in Fig. 10, and they were injected into female nude rats. *Arrow* indicates initiation of therapy. *A*, human melanoma cells were coinjected with m7E3 F(ab')$_2$ or vehicle control, and therapy was initiated on the same day. m7E3 F(ab')$_2$ at a dose of 6 mg/kg or vehicle control was injected either 3x/week or 5x/week. Each data point represents the mean of 6 animals/group; *bars*, ± SD. *B*, human melanoma cells were injected into nude rats, and antibody therapy was initiated 15 days post-tumor cell inoculation after tumors had reached a measurable volume of ~150 mm^3. Antibody or vehicle control was injected from day 15 until day 35 at a dose of 3 mg/kg (daily i.p. injections), and tumor volume measurements were recorded as described in "Materials and Methods." Each data point represents the mean tumor volume from 5 animals in the control and 4 animals in the m7E3 F(ab')$_2$-treated group; *bars*, ± SD. *C*, final weight of tumors excised on day 36 from experiment described in *B*. Bars represent the mean tumor weight from in the control (n = 5) and in the m7E3 F(ab')$_2$ (n = 4)-treated groups; *bars*, ± SD.

growth when compared with blockade of a single integrin receptor. c7E3 Fab, which binds with equivalent affinity to platelet GPIIb/IIIa and αvβ3, inhibited human melanoma and endothelial cell adhesion, migration, invasion, and lung colonization of human melanoma cells in nude mice. In addition, m7E3 F(ab')$_2$ inhibited angiogenesis and growth of human melanoma tumors *in vivo*. Collectively, our results suggest that c7E3 Fab and m7E3 F(ab')$_2$ with their multireceptor activity possess antiangiogenic and antimetastatic properties.

The requirement of platelets in hematogeneous spread of tumor cells was recognized almost 30 years ago and is reviewed in detail elsewhere (1, 31, 32). When metastatic tumor cells are shed into the blood circulation, they rapidly recruit platelets to form tumor cell-platelet aggregates, which results in a transient decrease in circulating platelet count (15, 33). Several preclinical animal models have demonstrated that blockade of platelet GPIIb/IIIa integrin inhibits lung colonization of tumor cells (15, 34). By blocking tumor cell-expressed αvβ3 integrin without inhibiting platelet function, c7E3 Fab, in this study, dramatically inhibited the metastatic ability of human melanoma cells in SCID mice. In this animal model, c7E3 Fab did not cross-react with mouse platelets; therefore, the results demonstrate that human melanoma cell-expressed αvβ3 integrin participates in lung metastasis.

In addition to facilitating metastasis, platelets can also stimulate tumor-induced angiogenesis. Platelet granules contain a variety of angiogenic factors such as VEGF that are rapidly secreted on platelet activation. Previous studies have revealed that an increase in platelet count is an indicator of poor prognosis in patients with lung and ovarian carcinoma (18–20), and platelet-secreted VEGF is inversely correlated with survival of patients with cancer (35). Pinedo *et al.* (12) have postulated that a true antiangiogenic agent must target platelets, but direct evidence to support this hypothesis is lacking. Our data provided novel evidence to support this hypothesis and demonstrate that platelets stimulated endothelial sprouting *in vitro*, and c7E3 Fab inhibited this sprouting. Earlier studies demonstrated that c7E3 Fab inhibited secretion of VEGF from platelets (15, 16); therefore, it is conceivable that VEGF could be contributing to platelet-stimulated endothelial cell sprouting. Platelet-secreted VEGF is probably not the only factor that stimulates angiogenesis, because platelets also contain other growth factors such as TGF-β and thrombin (13) that can stimulate endothelial cell sprouting. c7E3 Fab inhibits platelet-endothelial binding (36) and secretion of platelet granules containing growth factors (13), which may explain why c7E3 Fab completely blocked platelet-stimulated endothelial cell sprouting. This is an important finding, because it demonstrates that not just tumor cells, but host cells can contribute to tumor angiogenesis.

In addition to blocking platelet GPIIb/IIIa, abciximab also inhibits αvβ3 function. Because αvβ3 is an essential receptor for angiogenesis, c7E3 Fab can inhibit endothelial cell proliferation, adhesion, migration, invasion, and induce apoptosis of proliferating cells. Human melanoma cell-expressed αvβ3 participates in cell adhesion, migration, and invasion, and increase in β3 integrin inversely correlates with survival of melanoma patients (9, 10). c7E3 Fab completely inhibited αvβ3-mediated human melanoma cell adhesion, spreading, and invasion. More importantly, m7E3 F(ab')$_2$ has direct antitumor activity *in vivo*. Blockade of human melanoma cell-expressed αvβ3 by m7E3 F(ab')$_2$, without blocking host cell integrin, resulted in a partial inhibition of tumor growth in nude mice. Interestingly, combined blockade of host integrins (platelet GPIIb/IIIa and endothelial αvβ3) and tumor cell-expressed αvβ3 completely prevented tumor formation and growth in nude rats. In this rat xenograft model, which mimics the clinical situation, combined antiangiogenic and antitumor activity of m7E3 F(ab')$_2$ was superior at inhibiting tumor growth when compared with its antitumor activity in the mouse xenograft model.

Tumor growth and angiogenesis involves multiple integrin receptors; therefore, monospecific αvβ3 antagonists may not be effective at inhibiting tumor progression. Agents that block multiple integrin receptors may be more effective at inhibiting tumor growth and angiogenesis. This study provides novel evidence that combined inhibition of αvβ3 and GPIIb/IIIa may be an effective approach to inhibiting tumor growth, angiogenesis, and metastasis.

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